Clonal genetic evolution at relapse of favorable-risk acute myeloid leukemia with *NPM1* mutation is associated with phenotypic changes and worse outcomes

Carmen Martínez-Losada,[1] Juana Serrano-López,[1] Josefina Serrano-López,[1] Nelida I. Noguera,[2,3] Eduardo Garza,[4] Liliana Piredda,[2] Serena Lavorgna,[2] María Antonietta Irno Consalvo,[2] Tiziana Ottone,[2] Valentina Alfonso,[2] Juan Ramón Peinado,[5] María Victoria Garcia-Ortiz,[6] Teresa Morales-Ruiz,[6] Andrés Jérez,[7] Ana María Hurtado,[7] Pau Montesinos,[8] José Cervera,[8] Esperanza Such,[8] Marian Ibañez,[8] Amparo Sempere,[8] Miguel Ángel Sanz,[8] Francesco Lo-Coco[2,3] and Joaquín Sánchez-García[1]

[1]Hematology Department, Reina Sofía University Hospital/Maimonides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba, Spain; [2]Department of Biomedicine and Prevention, "Tor Vergata" University, Rome, Italy; [3]Laboratory of Neuro-Oncohematology, Santa Lucia Foundation, Rome, Italy; [4]Laboratorios Dr. Moreira, Monterrey, Mexico; [5]Medical Sciences Department, Faculty of Medicine/University of Ciudad Real (UCLM), Spain; [6]Maimónides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba/ Reina Sofia University Hospital, Spain; [7]Hematology Department University Hospital Morales Meseguer-IMIB and [8]Hematology Department, Hospital Universitari I Politècnic La Fe, Valencia, Spain

Correspondence: juanitatolea@yahoo.es
doi:10.3324/haematol.2018.188433

SUPPLEMENTARY METHODS

Bone marrow-derived genomic DNA was obtained from paired diagnosis-relapse samples using QIAmp® DNA Mini Kit, (Qiagen, Valencia CA) following manufacturer's instructions. The quality and concentration of extracted DNA were assessed by spectrophotometry by Nano-Drop_1000 (NanoDrop Technologies Inc, Wilmington, DE).

NPM1^{mut} was routinely detected at diagnosis and relapse by high-resolution-melting curves or restriction fragment length polymorphisms (N=3), *RUNX1-RUNX1T1* and *CBFB-MYH11*CBF by standardized RT-PCR and *CEBPA* by Next Generation Sequencing. Relapses after allo-SCT were all from recipient origin as assessed by routine chimerism analysis. *FLT3-ITD* and *TKD* mutations were screened at diagnosis and relapse by Sanger Sequencing (N=3), NGS (N=6) or multiplex PCR and capillary electrophoresis assay (N=17).

1. Next Generation Sequencing (NGS)

Specific primers flanking positions of interest were designed. PCR reaction was done with 10 ng of DNA as follows: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds; 60°C for 10 seconds; 72°C for 30 seconds and a final elongation step at 72°C for 30 seconds. PCR products were prepared for sequencing using the NEXTflex™ Rapid DNA Sequencing Kit (Bio Scientific, Austin TX), following the manufacturer's instructions. Three independent PCR products of suspected cases and control samples were used for sequencing. The resulting libraries were sequenced on an Illumina Hi-Seq 1500 at 2 x 100. Primary analyses, including base calling, read filtering and demultiplexing were performed according to the standard Illumina processing pipeline (CASAVA 1.8.2). Sequence read pairs were mapped to the human genome assembly GRh37/hg19 (build 37.2, February 2009) with BWA (Untergasser *et al*, 2012). SAM tools were used to calculate read depth and nucleotide frequencies for

each position of the amplicons (Untergasser *et al*, 2012; Li H *et al*, 2009). Median coverage was 1361 (range 367-4000).

2. DNA sequencing analysis by Sanger

To perform Sanger sequencing method, we used 20 ng of total genomic DNA to perform PCR (AmpliTaqGold® with GeneAmp®. Applied Biosystems. Roche. New Jersey. USA). PCR products were visualized under UV light lamp to confirm the presence of the correct size of fragments. Then, amplicons were purified using QIAquick® PCR Purification/ Gel Extraction KIT (QIAGEN Valencia CA. USA) and DNA sequencing analysis was executed bidirectional by an internal sequencing primer (BigDye® terminator v3.1 Cycle Sequencing kit, Applied Biosystems Inc., Foster City, CA). Sequencing PCR protocol consisted of an initial cycle at 96ºC for 1 minute followed by 40 cycles at 96ºC for 10 seconds, 50ºC for 5 minutes and 60ºC for 4 seconds. Then, products were purified using Centri-SEP Spin Columns, Princeton Separation KIT (Applied Biosystems Inc., Foster City, CA). Sequencing data were analyzed by Seq Scanner ® V1.0 software (Applied Biosystems Inc., Foster City, CA).

To analyze specified mutations in *KRAS, NRAS, TP53, DNMT3A, IDH1 and IDH2* genes, we designed a pair of normal desalted primer sequences per gene using Internet-based software Primer3web version.4.0.0. (Li H *et al*, 2009) Then, we completed Basic Local Alignment Search Tool (https://www.ncbi.nlm.nih.gov/refseq/rsg/) to address the optimal alignment of sequences. All primers sequences are described in the table below.

3. SNP and deletion analysis by pyrosequencing and homology modeling of *DNMT3A* new mutations.

To demonstrate the leukemic specificity of two new mutations found in DNMT3A gene, we analyzed by pyrosequencing normal BM cells from healthy donors and samples at complete remission from carrier patients. Primers for pyrosequencing

based allele quantification analysis were designed using the PyroMark Assay Design Software 2.0.1.15 (Qiagen), with the forward being 5' biotinylated. A 178 base pairs (bp) region was amplified on human genomic DNA. PCR reaction was performed in 30 µL of 0.05 µmol/L specific primers, 0.2 mmol/L dNTPs, 50 ng genomic DNA, 1.5 mmol/L magnesium chloride, 1× PCR buffer, 0.2 U of Immolase DNA polymerase (Bioline) for 45 cycles with annealing temperature of 56ºC. PCR products were verified by electrophoresis on a 2% w/v agarose gel. Pyrosequencing was completed using a PyroMark Q24 pyrosequencer (Qiagen, Valencia CA) with 10 to 15 ul of PCR product according to the manufacturer's instructions. The allele and deletion frequencies in the target region were analyzed by PyroMark Q24 software version 2.0.6.

4. Homology modeling

A three-dimensional model of mutated DNA (cytosine-5)-methyltransferase 3A was calculated using Swiss-Model, a semi-automated modeling server, and analysed with the Swiss-Pdb Viewer. The amino acid sequence of the mutated protein was compared with the sequences of the protein structures deposited in the Protein Data Bank. After analyzing structures of proteins that had the highest structural quality and significant sequence similarity with the mutated protein, we chose 3a1b.1.A and 4u7p.1.A as templates. The templates were superimposed and aligned structurally. The quality of the resulting models was verified manually with Swiss-Pdb Viewer. The figures were rendered with POV-Ray.

5. Phenotypic analysis by Multiparameter Flow cytometry

Immunophenotype analysis of leukemic cells was performed on erythrocyte-lysed bone marrow samples obtained at diagnosis and relapse by multiparametric flow cytometry (MFC)[22]. At least 1 x 10^6 cells were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin-chlorophyll-protein (PerCP) or allophcocyanin (APC) conjugated Monoclonal-Antibodies combinations which included: anti-CD65 (clone 88H7), anti-CD15 (clone HI9), anti-HLADR (clone L243), anti-CD7 (clone 4H9), anti-CD2 (clone S5.2), anti-CD14 (clone MΦP9), anti-CD19 (clone J3-119), anti-CD117 (clone 104D2), anti-CD56, anti-CD13 (clone L138), anti-CD11b (clone ICRF44), anti-CD38 (clone HB-7), anti-CD34 (clone 8G12) and anti-CD33 (cloneWM53), mostly from Becton-Dickinson).

At least 30.000 leukemic events were acquired mostly in FACSCalibur or FACSCanto II dual-triple laser flow cytometers and list modes files were analyzed with CELLQUEST™, FACSDIVA™ or PAINT-A-GATE Software. (BD, Biosciences). Antigen expression was categorized considering mean value at Log-scale as "strong" (greater

than 10^4), “dim” (between 10^3 and 10^4) and “negative” (lower than 103, as matched isotype controls). Multidimensional analyses of immunophenotypes obtained at diagnosis and relapse were performed using the File merge and Automatic Population Separator (APS) functions of the Infinicyt software (Cytognos SL, Salamanca, Spain).

6. Statistical analysis

The Fisher exact test or chi-square test (with Yates’s correction for continuity when needed) was used to compare categorical data and quantitative data was compared with the Mann-Whitney U test. Overall survival (OS) from leukemia relapse was calculated using the Kaplan-Meier method including the 95% confidence interval (95% CI). Clinical status was updated in February 2017. The two-sided log-rank test was used for univariate comparisons. Multivariate analysis was performed by Cox regression forward stepwise method. Calculations were performed using IBM SPSS statistical for Windows version 17.0. (IBM Corp., Armonk, NY, USA).

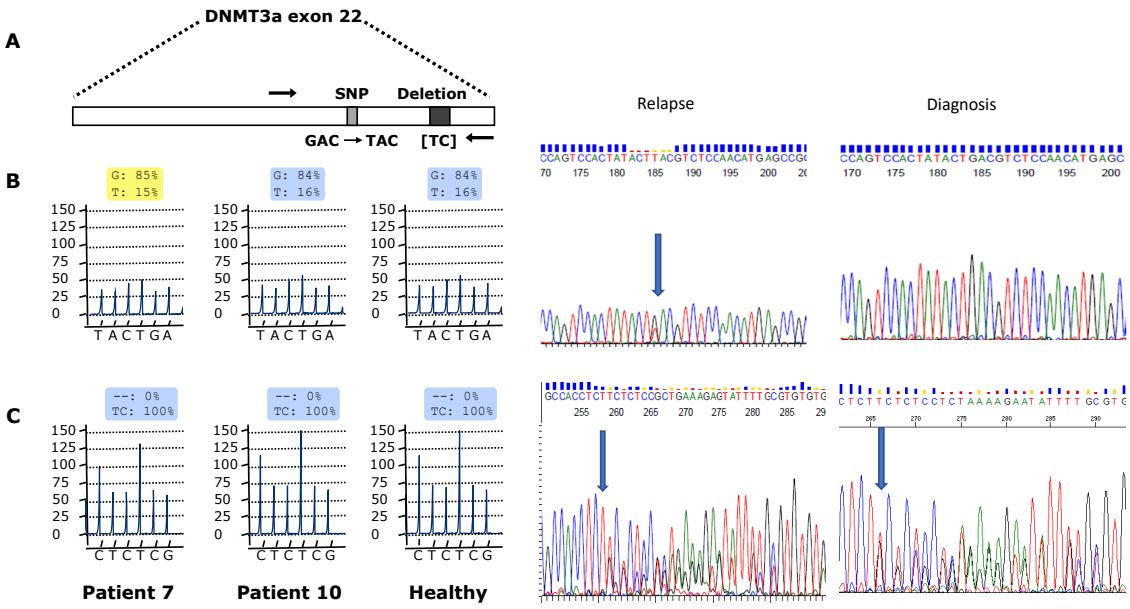
7.- Primer sequences

GENE	PRIMER NAME	SEQUENCE 5´- 3´
NRAS _exon1	Sequence amplification forward (FW)	CGGTGTTTTTGCGTTCTCTA
NRAS_exon1	Sequence amplification reverse (RV)	TTGCATAACTGAATGTATACCCAAA
NRAS_exon1	Internal sequencing FW	GATGTGGCTCGCCAATTAAC
NRAS_exon1	Internal sequencing RV	TGGGTAAAGATGATCCGACA
NRAS_exon2	Sequence amplification FW	CAATGTCAAACAACCTAAAACCA
NRAS_exon2	Sequence amplification RV	GGCAGAAATGGGCTTGAATA
NRAS_exon2	Internal sequencing FW	TTGCATTCCCTGTGGTTTTT
NRAS_exon2	Internal sequencing RV	TGGTAACCTCATTTCCCCATA
KRAS_exon1	Sequence amplification FW	CTTAAGCGTCGATGGAGGAG
KRAS_exon1	Sequence amplification RV	CCCTGACATACTCCCAAGGA
KRAS_exon1	Internal sequencing FW	AAAAGGTACTGGTGGAGTATTTGA
KRAS_exon1	Internal sequencing RV	AGAATGGTCCTGCACCAGTAA
KRAS_exon2	Sequence amplification FW	ATCCTAATGGGTATGTGGTAGCAT
KRAS_exon2	Sequence amplification RV	AAGAGTACAGAAGGCTGTGGAGTC
KRAS_exon2	Internal sequencing FW	GGTGCTTAGTGGCCATTTGT
KRAS_exon2	Internal sequencing RV	TGCATGGCATTAGCAAAGAC
TP53_exon4_5	Sequence amplification FW	AAAAAGAAAAGCTCCTGAGGTGAG
TP53_exon4_5	Sequence amplification RV	CCCATTTACTTTGCACATCTCA
TP53_exon4_5	Internal sequencing FW	GGAGGTGCTTACGCATGTTT
TP53_exon4_5	Internal sequencing RV	GGGAGGTCAAATAAGCAGCA
TP53_exon6	Sequence amplification FW	TGTGCAAAGTAAATGGGTTTAACTA
TP53_exon6	Sequence amplification RV	AAGAAAACTGAGTGGGAGCAGTAAG
TP53_exon6	Internal sequencing FW	AAAAAAGGCCTCCCCTGCTTGCCAC
TP53_exon6	Internal sequencing RV	GATGTGATGAGAGGTGGATGGGTA
TP53_exon7_8	Sequence amplification FW	ACTCAGTTTTCTTTTCTCTGGCTTT
TP53_exon7_8	Sequence amplification RV	CAAATGCCCCAATTGCAGGTAAAACA
TP53_exon7_8	Internal sequencing FW	CTTAGGCTCCAGAAAGGACAAG
TP53_exon7_8	Internal sequencing RV	CAAATGCCCCAATTGCAGGTAAAACA
DNMT3A	Sequence amplification FW	TTTATAAAGGACAGAAGATTCGGCAGAA
DNMT3A	Sequence amplification RV	CCTTACACACACGCAAAATACTCCTT
DNMT3A	Internal sequencing FW	TTTATAAAGGACAGAAGATTCGGCAGAA

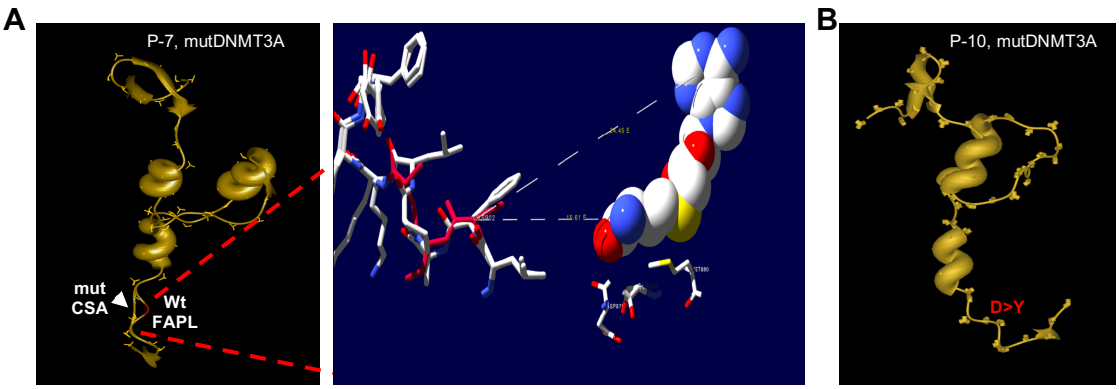
DNMT3A	Internal sequencing RV	CCTTACACACACGCAAAATACTCCTT
IDH 1	M13_Sequence amplification FW	TGTAAAACGACGGCCAGTTGAGAAGAGGGTTGAGGAGTT
IDH_1	M13_Sequence amplification RV	CAGGAAACAGCTATGACCAACATGCAAAATCACATTATTGCC
IDH_1	M13_Internal sequencing FW	TGTAAAACGACGGCCAGT
IDH_1	M13_Internal sequencing RV	CAGGAAACAGCTATGACC
IDH_2	M13_Sequence amplification FW	TGTAAAACGACGGCCAGTGGGTTCAAATTCTGGTTGAA
IDH_2	M13_Sequence amplification RV	CAGGAAACAGCTATGACCTAGGCGAGGAGCTCCAGT
IDH_2	M13_Internal sequencing FW	TGTAAAACGACGGCCAGT
IDH_2	M13_Internal sequencing RV	CAGGAAACAGCTATGACC

SUPPLEMENTAY FIGURES

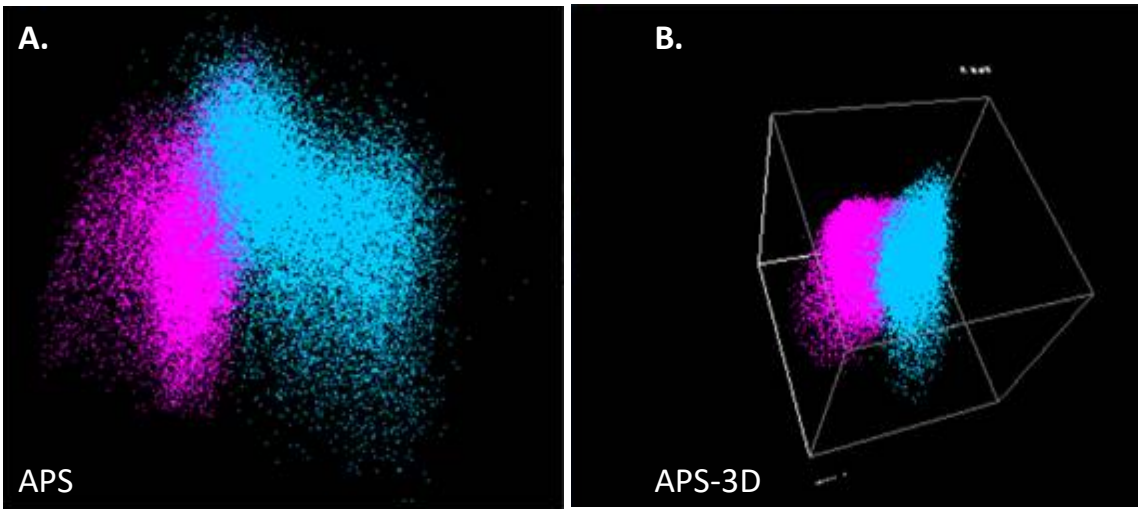
Supplementary Figure 1. Analysis of sequence variations in DNMT3a exon22 from patients 7 and 10 by pyrosequencing and Sanger sequencing. (A) Map of DNMT3a exon 22 showing the analyzed region (black arrows) for the substitution c.2626 G>T and the deletion c. 2705_2706delTC, respectively. (B) Left: Determination of allele frequency of single nucleotide polymorphism c.2626 G>T by pyrosequencing. Right: Determination of C.2626 G>T mutation by Sanger: the mutation is only found in the relapse sample. (C) Left: Deletion c.2705_2706delTC percentage analysis by pyrosequencing. Right: Determination of c.2705_2706delTC by Sanger: the mutation is found in the diagnosis and relapse samples. A healthy donor was included in the experiment.



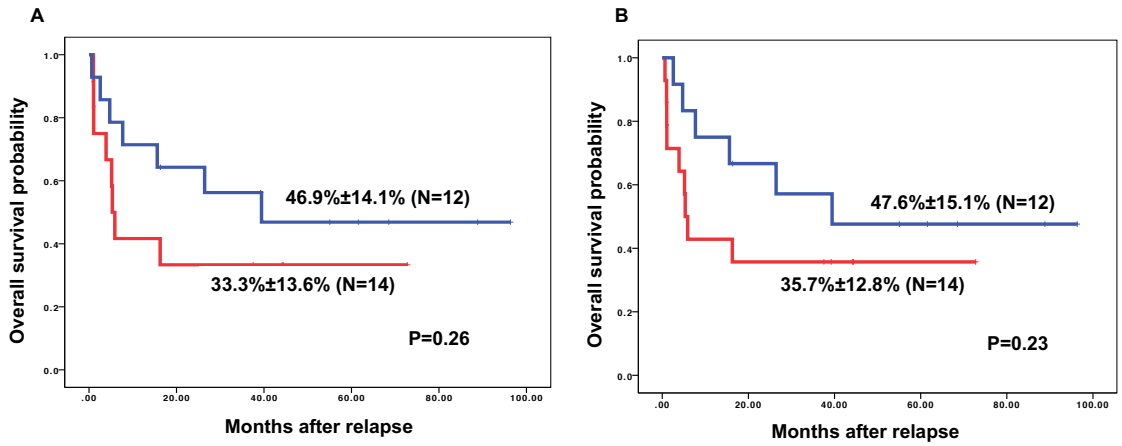
Supplementary figure 2. Three-dimensional models for DNMT3A mutants. The amino acid sequence of the mutated DNMT3A was compared with the wild type sequences from Protein Data Bank. (A) Deletion of two nucleobases in DNA introduced small changes in amino acid sequence (left) although far from active side (center). (B) Changes in one nucleobase produce an amino acid substitution and non-sequence alteration were found in 3D superposition.



Supplementary Figure 3. Representative case (Patient 10) of multidimensional analyses of complete immunophenotype profile using Automatic Population Separator (APS) function of Infinicyt software. Pink population corresponds to diagnosis and blue population corresponds to the relapse.



Supplementary Figure 4. Overall Survival. (A) patients with (red) or without (blue) phenotypic changes and (B) patients with (red) or without (blue) phenotypic and/or genetic changes.



SUPPLEMENTARY TABLES.

Supplementary Table 1.
Clinical and biological data comparing genetic patterns at relapse

	No clonal evolution N (%)	Clonal evolution N (%)	P Value
Number of patients	20	6	
Gender (Male)	11 (55)	2 (33,3)	NS
Median (range) WBC x10^9/l	17 (1.49-149)	16.6 (4-145)	NS
Median (range) marrow blasts	63 (32-100)	71 (52-92)	NS
Cytogenetic / molecular group			
-NK-NPM1mut	10 (50)	6 (100)	NS
-t(8;21)	4 (20)	0	
-inv(16)	4 (20)	0	
-NK-CEBPAbi	2 (10)	0	
SCT before relapse			
-Allogeneic	6 (30)	4 (66,7)	NS
-Autologous	7 (35)	0	
Phenotypic changes	8 (40)	4 (66.7)	NS
Median months until relapse	18.5 (6-252)	12 (6-67)	NS
Allo-SCT after relapse	11 (55)	1(16.7)	NS
OS Probability	48.5±11.5%	16.7±17.9%	**.003**

WBC: White BLood cell; NK: normal karyotype; SCT: Stem cell transplantation; OS: Overall Survival

Supplementary Table2.
Multivariate Cox regression analysis of variable influencing Overall Survival alter leukemia relapse

Variable (categories and codes)	***Overall survival***		
	Beta coefficient (SE)	Hazard ratio (95% CI)	P value
Allo-SCT as treatment for relapse (Yes,No)	- 0.28 (.80)	0.06 (0.01 – 0.30)	0.001
Clonal evolution (Yes, No)	1.60 (.64)	4.99 (1.43 – 17.43)	0.01
Age at relapse, yr (Continuous)	-	-	0.907
Relapse Free Survival (Continuous)	-	-	0.339
Previous Allo-SCT at first CR (Yes,No)	-	-	0.236
Phenotypic shifts (Yes,No)	-	-	0.242
Genetic subgroup (*NPM1*^{mut}, CBF/CEBPA)	-	-	0.483

Supplementary Table3.
Karyotype at diagnosis and relapse

Patient	Karyotype at diagnosis	Karyotype at relapse
1	46XY	46XY
2	46XX	NA
3	46XX	46XX
4	46XX	46XX
5	46XY, t(8;21)	46XY, t(8;21)
6	46XY,t (8;21)	NA
7	46XX	46XX
8	46XY	NA
9	46XY	NA
10	46XX	46XX
11	46XY	NA
12	46XY,inv16	46XY,inv16
13	46XY	46XY
14	46XX	46XX
15	46XY	46XY
16	46XX	46XX
17	46XX,t (8;21)	NA
18	46XY,t (8;21)	NA
19	46XX	NA
20	46,XX, inv16	46,XX, inv16
21	46XY	46XY
22	46XX	46XX
23	46XY,inv16	46XY,inv16
24	46XX,inv16	46XX,inv16
25	46XY	46XY
26	46XX	46XX

NA: Not available.