

Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic *RUNX1/AML1* lesions

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ABSTRACT

Fanconi anemia (FA) is a genetic condition associated with bone marrow (BM) failure, myelodysplasia (MDS) and acute myeloid leukemia (AML). We studied 57 FA patients with hypoplastic or aplastic anemia (n=20), MDS (n=18), AML (n=11), or no BM abnormality (n=8). BM samples were analyzed by karyotype, FISH, high-density CGH/SNP-arrays with respect to paired fibroblasts, and by selected oncogene sequencing. A specific pattern of chromosomal abnormalities was found in MDS/AML, which included 1q+ (44.8%), 3q+ (41.4%), -7/7q (17.2%), and 11q- (13.8%). Moreover, cryptic *RUNX1/AML1* lesions (translocations, deletions or mutations) were observed for the first time in FA (20.7%). Rare mutations of *NRAS*, *FLT3-ITD*, *MLL-PTD*, *ERG* amplification, and *ZFP36L2-PRDM16* translocation, but no *TP53*, *TET2*, *CBL*, *NPM1* and *CEBP α* mutations were found. Frequent homozygosity regions were related not to somatic copy-neutral loss-of-heterozygosity but to consanguinity, suggesting that homologous recombination is not a common progression mechanism in FA. Importantly, the *RUNX1* and other chromosomal/genomic lesions were found at the MDS/AML stages, except for 1q+ which was found at all stages. These data have important implications for staging and therapeutic managing in FA patients, and also to analyze the mechanisms of clonal evolution and oncogenesis in a background of genomic instability and BM failure.

INTRODUCTION

Fanconi anemia (FA) is a rare genetic condition characterized by congenital abnormalities, chromosome fragility, progressive bone marrow failure and cancer susceptibility.^{1,2} Thirteen FA genes have been identified to date, the product of which function in the FA/BRCA DNA-repair pathway.^{3,4} FA patients often, but not always, present with a combination of various congenital abnormalities like short stature, thumb and radius deformities, peculiar facies, cutaneous spots, and organ malformations. Most FA patients develop bone marrow failure throughout the course of the disease, usually during their first and second decades of life and, for the majority of patients, the suspicion of FA is only made after the onset of pancytopenia.^{1,2} There is a strong predisposition to hematologic and epithelial malignancies, with cumulative probabilities of an FA patient developing myelodysplasia (MDS) or acute myeloid leukemia (AML) being 30-40% by age 40 years.⁵⁻⁷ In a number of patients, the underlying diagnosis of FA is not known until a MDS/AML occurs. Biologically, MDS in FA often presents as refractory cytopenia with multilineage dysplasia, with or without excess of blasts.⁸ It is noteworthy that a level of dyserythropoiesis is almost constant in FA, and a mild dyserythropoiesis is not considered as a MDS criteria in this population. Acute leukemia can be diagnosed primarily or following a MDS phase with an increasing fraction of blast cells in the bone marrow. Cytogenetic abnormalities are frequently found, and translocations of chromosome 1q, monosomy 7, and gains of 3q have been reported.^{5,8-11} Hematopoietic stem cell transplantation is the current treatment in FA to cure aplastic anemia or MDS and to prevent transformation into leukemia.^{12,13} It has to be carefully considered upon clinical and biological criteria which include the age, the severity of the cytopenia, significant bone marrow dysplasia, excess of blast cells, cytogenetic abnormalities and immunological compatibility with the donor.

Here we investigated the pattern of chromosomal and genomic abnormalities in FA and their association to MDS/AML. We used high density DNA microarray and oncogene sequencing in addition to karyotype and FISH to analyze a series of FA patients at the different stages of bone marrow progression. We found, for the first time in FA, recurrent abnormalities of the oncogene *RUNX1/AML1* and included them in a highly recurrent pattern of abnormalities in FA. We also found that mutations of other common MDS/AML oncogenes and tumor suppressor genes were rare in FA, as were rarely detected acquired uniparental disomy (UPD) regions. Somatic reversion of the constitutional *FANCA* gene mutations was not found. Importantly the presence of chromosomal/genomic abnormalities was correlated to the stage of progression to MDS/AML, except for 1q+ which was found at all stages. These data have important implication not only for the cytogenetic staging of the bone marrow cells in FA patients but also as a basis to analyze the step-wise clonal selection and oncogenesis in

patients with hypoplastic bone marrow and genomic instability, with potential relevance for non-FA patients.

MATERIALS AND METHODS

Patients

Bone marrow cells from 57 FA patients were analyzed in this study. Patients and/or samples were referred between February 2002 and April 2010 to the French Center for Constitutional Bone Marrow Failure of Saint-Louis Hospital, Paris, France. Inclusions in the present study were retrospective, based on the availability of BM frozen cells and on an enrichment of the series in patients aged more than 18, and in patients with morphological and/or karyotype abnormalities on their BM aspirate follow up. All patients had a definitive FA diagnosis based on the usual biological FA criteria including chromosomal breakage test in blood, which were performed or reviewed at the Fanconi Anemia Laboratory at Saint-Louis Hospital.^{14,15} FA mutations were searched for in the patient DNAs (in most cases from primary fibroblasts) in collaboration with Oncogenetic Laboratory of Curie Institute, Paris, and mutation data have been sent to the Fanconi Anemia Mutation Database (www.rockefeller.edu/fanconi/mutate/). Consanguinity was defined according to the familial history and/or homozygous *FANC* gene mutation or deletion (when known). Informed consent for sample collection was obtained from the patients and/or their relatives according to the guidelines of the Tumorothèque of Saint-Louis Hospital following the Declaration of Helsinki, and the study was approved by the Institutional Saint-Louis Hospital review board.

Clinical characteristics of the 57 FA patients are shown in Table 1. Age was considered at the time of the cytogenetic analysis. Bone marrow categories were defined as follows. Aplastic anemia without MDS were diagnosed according to the criteria of the International Study of Aplastic Anemia and Agranulocytosis.¹⁶ Two sub-groups were further defined: aplastic anemia (AA) when BM cellularity was below 25% of the normal hematopoietic cellularity, and moderate hypoplastic anemia (MH) when BM cellularity ranged 25% to 50%, based on an evaluation on BM aspiration and smears (BM biopsies were not performed in these FA patients). MDS were classified according to the World Health Organization classification 2008 (Table 1). Of note a moderate dyserythropoiesis is usual in FA and is not considered as a criteria for MDS,⁸ and patients without other morphological abnormalities were classified as normal, MH, or AA depending of the BM cellularity. Acute leukemia was defined by BM blast cells > 20%. When several BM samples were obtained from the same patient, the most informative was considered (usually the most recent except technical issues).

Samples

Bone marrow samples were obtained from BM aspirate as performed in the systematic follow up of FA patients, or when a change happened (like the onset of a AML). Skin biopsies and primary fibroblast culture were performed at the initial FA diagnosis work-up as previously described.^{14,15} DNAs were extracted using the QIAamp DNA Mini Kit (Qiagen, www.qiagen.com). The GenomePlex Whole Genome Amplification kit WGA2 was used when necessary according to manufacturer's instructions (Sigma-Aldrich, France) before array-CGH processing.

Karyotype and FISH analyses

Cytogenetic analysis was performed using classical procedures. Images were acquired with the Metafer Slide Scanning System and classified on the Ikaros Karyotyping System (MetaSystems, Germany). Karyotypes were described according to International System for Human Cytogenetic Nomenclature (ISCN), 2008. At least 20 metaphases were screened in each patient, except in cases where abnormal mitoses were obtained and in 4 cases with mitosis failure.

Interphase and/or metaphase (when available) FISH were performed from cryopreserved BM pellets, using break-apart probes *RUNX1* (Amplitech, France) and *EVI1* (Kreatech, France). *PRDM16* flanking probes were obtained from Amplitech (France) using the BAC RP4-785P20 and RP11-333E3. At least 200 nuclei were scored per sample for each probe using an epifluorescence microscope Axio Imager 2 (Carl Zeiss, Germany) and the Isis imaging system (MetaSystems, Germany).

SNP-array and array-CGH analyses

All the 57 DNA bone marrow samples were analyzed by high density DNA-array technologies: 39 using SNP-arrays and 32 using array-CGH (14 with both technologies), see Supplemental Figure S1. For SNP-arrays, the Affymetrix Genome-Wide Human SNP-Array 250K (Nsp and/or Sty, n=26 patients) or 6.0 (n=13 patients) were used. Paired fibroblast DNAs were available for 36 patients analyzed in SNP-arrays. DNAs were processed for SNP-array using the manufacturer's recommendations (Affymetrix, Santa Clara, CA, USA). The arrays were scanned on the Affymetrix Scanner 3000 7G. CEL files produced by Genotyping Console software were imported into the Genomic Suite 6.5 software (Partek, www.partek.com) for genotyping and copy number analyses. Both SNP and CNV probes were used in the analysis. Significantly different regions were determined using the Hidden Markov Model and Segmentation algorithms to detect copy number (CN) changes and homozygosity regions. As a normal reference population we used the International HapMap project and individual references from the paired analysis of the fibroblast DNAs. Agilent Array-CGH technology (180K, n=31; 1M, n=1) was used in 32 BM samples DNAs following

the manufacturer's recommendations (Agilent Technologies, Santa Clara, CA, USA). The arrays were scanned using the SureScan High-Resolution Technology (Agilent) and the images were processed using Agilent Feature Extraction software applying linear and lowess normalization methods and local background subtraction. Analysis was performed using the Agilent Genomic workbench software, with the help of the ADM-2 algorithm. Array-CGH data were also imported and analyzed into the Partek Genomic Suite 6.5 software. For both the SNP-array and array-CGH technologies, the final retained abnormalities were validated by visual analysis by two separate investigators (S.Q. and J.S.) considering the size and Log₂ ratio of the abnormalities with respect to the individual background noise of each array at each particular chromosomal location, as we performed previously.^{17,18} Polymorphic copy number variations (CNV) were excluded using the paired analysis of BM and fibroblast DNAs, and using the Database of the Genomic Variants tracks in the UCSC Genome Browser or in the Genomic Workbench Agilent software. Final validated data are indicated in the Results, Tables and Figures. The UCSC Genome Browser was used for location of the genes on the human genome (www.genome.ucsc.edu). All original microarray data have been deposited in ArrayExpress under accession number E-MTAB-529.

Oncogenes and tumor suppressor genes sequence

Sequencing was performed on genomic DNA for *RUNX1/AML1*, *TET2*, *CBL*, *TP53*, *NRAS*, *NPM1*, and *CEBP α* genes, and for *FLT3* tyrosine kinase domain (*FLT3-TKD*), *FLT3* internal tandem duplication (*FLT3-ITD*) and MLL partial tandem duplication (*MLL-PTD*), as previously described.^{19,20}

RESULTS

A highly recurrent pattern of chromosomal gains and losses in FA bone marrow

We analyzed bone marrow (BM) samples from a series of 57 FA patients (Supplemental Figure S1). All patients had both a conventional karyotype and a high density genome-wide CGH/SNP-arrays analyses. Somatic chromosomal gains and losses were detected in n=35 patients out of 57 (Table 2; complete data are shown in Supplemental Table S1), with a highly recurrent profile (Figure 1). The most frequent abnormality was the gain of the long arm of chromosome 1 (1q+; n=19 out of 35 patients with abnormalities, 54.3%). The 1q gain was complete (n=11) or interstitial (n=8), the minimal region mapping from 1q23 to 1q32 (74.9 Mb) (Figure 1). Other recurrent abnormalities were the gain of the telomeric part of chromosome 3 (3q+, n=12 out of 35 patients with abnormalities, 34.3%), 21q abnormalities (n=8, 22.9%), partial or complete deletion of chromosomes 7 (-7/7q, n=5, 14.3%), 11q (11q-, n=5, 14.3%), 20q (20q-, n=4, 11.4%), 5q (5q-, n=3, 8.6%), 9p gain (9p+, n=3, 8.6%), and trisomy 8 (+8, n=2, 5.7%). The 3q gain was telomeric in all cases, the 41.5 Mb minimal duplicated region mapping from 3q25 to 3q29 as described by Tonnie and al.¹¹ This region included the *EVI1* oncogene; break-apart FISH probes of the *EVI1* locus detected 3 bi-color signals related to 3q+ when expected from the CGH/SNP-arrays data but did not evidenced any direct, balanced or unbalanced, translocation (n=34 tested patients, data not shown).

Frequent regions of homozygosity in FA patients are related to consanguinity but very rarely to acquired uniparental disomy (UPD)

SNP-array analysis using paired DNAs from bone marrow and fibroblasts allows high-resolution detection of the acquired uniparental disomy (UPD, also called copy-neutral loss of heterozygosity, CN-LOH) in addition to copy-number changes.²¹ SNP-array analysis was performed in samples from 39 patients, including 36 with a paired BM+fibroblast analysis. In 4 patients only, a region of somatic UPD was present (Figure 1 and Supplemental Table S1), indicating that this type of genomic abnormality is rare in clonal evolution of FA. Noteworthy, a number of patients (n=11) had highly frequent regions of constitutive homozygosity as shown by fibroblast versus hapmap SNP-array analysis (Figure 2A). This feature was related to consanguinity, as expected from the familial history and homozygous constitutive *FANCA* mutations (Table 1). In these cases (all *FANCA*), the *FANCA* locus at 16q24 was included in a region of constitutive homozygosity (Figure 2A). Noteworthy, in one patient, a large region of acquired interstitial UPD in chromosome 13q was detected in the bone marrow cells and encompassed a shorter region of constitutive homozygosity, as demonstrated by the comparative paired analysis (Figure 2B).

Recurrent *RUNX1/AML1* genomic abnormalities in MDS/AML

RUNX1/AML1 translocations or mutations were initially found by CGH/SNP-arrays and gene sequencing analyses in n=5 patients (Supplemental Table S1). Therefore, we performed a systematic FISH analysis using *RUNX1* locus break-apart probes in n=34 patients of the series. This confirmed the array-based data by showing unbalanced translocations in 2 cases (EGF036 and EGF111) and a large deletion in 1 case (EGF203), whereas as expected the short deletion/mutations were undetectable by FISH (EGF117 and EGF171). Moreover, the break-apart FISH screen unrevealed an additional *RUNX1* locus abnormality in a sixth FA patient (EGF201), that is a cryptic balanced translocation which had not been previously detected by conventional karyotype (20 mitoses without abnormalities) nor by CGH/SNP-arrays (Supplemental Table S1).

Altogether, the *RUNX1* abnormalities were as follows (Figure 3): 3 focused deletions and a 7bp insertion, two unbalanced translocations including a t(1;21)(p36;q22)/*RUNX1-PRDM16* (evidenced by a *RUNX1* FISH signal at 1p36 and a split of the *PRDM16* probes), and in a sixth FA patient a balanced *RUNX1* translocation. Apart from the patient with the *RUNX1-PRDM16* translocation, insufficient material did not allow further investigation of the *RUNX1* partner region in the two other translocations (only *PRDM16* could be excluded by FISH in case EGF201). Importantly, all the 6 FA patients with *RUNX1* genomic abnormalities had severe MDS or AML.

Rare mutations of common MDS/AML oncogenes and tumor suppressor genes in FA

In 25 patients with sufficient tumor DNA material, including 20 with a clonal population evidenced by CGH/SNP-arrays (17 with MDS or AML), we searched for mutations of a set of cancer genes which are commonly targeted in MDS and/or AML, namely *TET2*, *CBL*, *NRAS*, *TP53*, *RUNX1*, *CEBP α* , *NPM1*, *FLT3-TKD*, *FLT3-ITD*, and *MLL-PTD*.^{19,22-26} A few mutations were detected: *MLL-PTD* in 2 cases, and *FLT3-ITD*, *RUNX1* and *NRAS* mutations in 1 case each (Supplemental Table S1). No mutation was detected by sequencing *TP53*, *TET2*, *CBL*, *CEBP α* , *FLT3-TKD* and *NPM1*. In the two patients with *MLL-PTD*, the copy number gain within the *MLL* gene was also detected by CGH/SNP-arrays, as expected (Supplemental Table S1).

In an additional AML case (patient EGF068), we found a 4.4 Mb amplification at 21q22 by array-CGH, which included not the *RUNX1* but the *ERG* oncogene (Supplemental Figure S2). In another patient with a RAEB-1, we detected an unbalanced t(1;2)(p36;p21) leading to the translocation of the *PRDM16/MEL* gene to the *ZFP36L2* locus as mapped by array-CGH and confirmed by *PRDM16* FISH (patient EGF037, Supplemental Figure S2). Such abnormalities have been previously reported in rare non-FA MDS, AML or blast crisis of

myeloproliferative disorder.²⁷⁻²⁹ Of note, the karyotype detected no chromosomal abnormality in patient EGF068, the *ERG* amplification as evidenced by array-CGH being cryptic and the sole abnormality found in this patient.

Chromosomal and genomic abnormalities, especially 3q+, 7/7q-, and *RUNX1* abnormalities, but not 1q+ and 20q-, are associated with MDS/AML

Bone marrow morphology of the FA patients of the present series ranged from normal FA morphology (n=8), medullary hypoplasia or aplastic anemia (MH/AA, n=20), MDS of various WHO 2008 subtypes (n=18), to AML (n=11). The vast majority of the chromosomal and genomic abnormalities were found in the 29 patients with MDS or AML (112 out of 130 abnormalities, *P* value <10⁻⁶; see also Table 2 and Figure 4). An important exception to this feature was the frequent gain of chromosome 1q; indeed, 1q+ was found at all stages of FA bone marrow evolution, i.e. normal (3/8 cases), MH/AA (3/20), MDS (7/18) and AML (6/11) (*P* value of Normal/MH/AA versus MDS/AML non significant, Table 2). A similar trend was found for 20q-, as expected from what is known in non-FA MDS.³⁰ By contrast, gain of 3q, deletion 7/7q and *RUNX1* abnormalities were strictly found in the MDS/AML patients (Table 2 and Figure 4). Specifically, 3q+ was found in 7/18 MDS (mainly high grade) and 5/11 AML; -7/7q in 1/18 MDS (this last being a RAEB-1) and 4/11 AML; and *RUNX1* abnormalities in 2/18 MDS (both AREB-1) and 4/11 AML, demonstrating the strong association of these 3 abnormalities with high grade MDS and blastic transformation (Table 2 and Figure 4). Of note, an isochromosome 7q (7p-/7q+), found in one patient with aplastic anemia, was not included in the -7/7q category. Deletion of 5q was observed in 3 MDS cases, one of them also displaying the only deletion 17p of this series, and trisomy 8 in two patients (1 MDS and 1 AML, both with a 1q+). As mentioned above, a 4.4 Mb amplification at 21q22 including *ERG* was found as a unique abnormality in an AML case with a normal karyotype, and a translocation t(1;2) targeting *PRDM16* was found in an AREB-1 (Supplementary Figure S2).

Altogether, 27 out of 35 patients with chromosomal/genomic abnormalities had MDS/AML, and this value raised to 27 out of 28 patients by keeping only the abnormalities other than 1q+ and 20q-. We then analyzed the association of the recurrent chromosomal or molecular abnormalities. Frequent combinations were 1q+/3q+, 1q+/3q+/7q-, 1q+/3q+/*RUNX1*, 1q+/3q+/11q-/*RUNX1*, or even in one patient 1q+/3q+/7q-/11q-/*RUNX1*. Noteworthy, one patient with AML had no detectable gross chromosomal abnormality but the combination of *RUNX1*, *NRAS* and *MLL-PTD* mutations, consistent with what was reported in non-FA patients,²⁶ another one with AML had 1q+, a fusion *ZFP36L2-PRDM16* and *FLT3-ITD*, and another one had 1q+,+8 and *MLL-PTD* with UPD11 (Figure 4 and Supplemental Table S1). These combinations highlight the multi-step oncogenic pathways in MDS/AML of FA patients.

No reversion mutation was found in the MDS/AML in FA patients

Acquired genetic reversion of the *FANC* gene mutations were reported in blood lymphocytes³¹ but also in an AML cell line from an FA *FANCD1/BRCA2*-mutated patient,³² suggesting that this phenomenon might be frequent and play a role in FA-related MDS/AML oncogenesis. Constitutional *FANC* genes mutations in the present series were initially identified in the primary fibroblast DNAs for the vast majority of the cases (J.S. and D.S.L., unpublished data). We re-sequenced these mutations in the bone marrow DNA of 20 patients with sufficient DNA and a clonal population on CGH/SNP-array data. No reversion event could be found in the present series, suggesting that *FANC* genetic reversion (somatic mosaicism) is not a common progression event in MDS/AML, at least in this cohort of FA patients mainly of the FA-A group.

DISCUSSION

To evaluate the pattern of chromosomal and genomic abnormalities in FA and its association with the stages of bone marrow progression, we used high resolution chromosomal and molecular techniques on bone marrow samples from a series of FA patients. This series was enriched in patients older than 18 year-old, and in patients with morphological or karyotypic abnormalities on the follow-up BM aspirate. Most patients (84.2%) were from the FA-A group, which is representative of French patients. We found a complex and highly recurrent pattern of chromosomal and genomic abnormalities. It included chromosome abnormalities previously reported in FA karyotypes^{5,8-11} like -7/7q, 3q+ and 1q+ and, for the first time in FA, cryptic abnormalities of the *RUNX1/AML1* gene at 21q and rare mutations or translocations of other known oncogenes. The vast majority of the chromosomal abnormalities were due to unbalanced translocations leading to gain or deletion of gross chromosomal regions, often telomeric (Figure 1). It is likely that these result from FA-related unresolved DNA damage during S phase which ultimately leads to double strand breaks and error-prone repair by non-homologous end joining (NHEJ). Indeed NHEJ is largely efficient in FA cells in contrast with homologous repair (HR) which is impaired.^{4,33,34} Moreover, few somatic copy-neutral loss of heterozygosity (UPD) regions were observed (Figure 1 and Supplemental Table S1), suggesting that HR is a rare mechanism of genetic progression in FA, in contrast to what is seen in non-FA MDS/AML patients.^{35,36} We also searched for genetic reversion events (somatic reversion of the constitutional FA gene mutation), a phenomenon which leads to somatic mosaicism in FA lymphocytes,³¹ considering what was found in an AML cell line of the D1/BRCA2 group.³² No *FANCA* reverse mutation was observed by re-sequencing the genomic DNA from the clonal BM cells in this series, suggesting that simple genetic reversion is not a frequent progression event in the majority of FA patients. Looking for more complex reversion mechanisms would need cellular testing of the BM clonal cells.

FA patients experience a high selective pressure to develop clonal MDS and AML during their teens or young adulthood. This is probably directly related to the cellular defects of FA cells, i.e. genomic instability and excess of cell death, together with a possible intrinsic hematopoietic stem cell defect and related bone marrow failure.^{13,37-40} With respect to aplasia and MDS/AML in non-FA patients,^{16,21,22,35,36} two chromosomal abnormalities were specifically associated to FA patients, 1q+ and 3q+. The 1q minimal duplicated region was very large (74.9 Mb) and contained many possible candidate oncogenes. For 3q, an obvious candidate oncogene within the large 41.5 Mb minimal duplicated region was *EVI1*.⁴¹ A focused *EVI1* amplification was reported previously in AML cell lines derived from a FA patient of the particular D1/BRCA2 downstream group.⁴² Here, break-apart FISH found frequent *EVI1* duplication, as expected from the CGH/SNP-array data, but no direct involvement in a fusion gene resulting from a translocation. Therefore the potential role of

EVI1 in the MDS/AMLs with 3q+ in typical FA-core patients remains elusive. The other recurrent chromosomal and genomic abnormalities we found can also be seen in non-FA patients with MDS/AML. Molecular oncogenic significance of the deletions 7q and 11q has not been elucidated to date, by contrast of the UPD7q and UPD11q which were recently associated to *EZH2* and *CBL* mutations, respectively.⁴³⁻⁴⁵ Importantly, we found targeted abnormalities of the *RUNX1/AML1* oncogene in several FA MDS/AML patients, i.e. unbalanced or balanced translocations including *PRDM16-RUNX1* fusion and inactivating mutations or focused deletions (Figure 3), reminiscent of *RUNX1* oncogenic lesions in non-FA patients.²⁵ The *RUNX1* protein is part of the CBF heterodimeric transcription factor, which regulates different levels of embryonic and adult hematopoiesis. A range of *RUNX1* genomic abnormalities were reported in non-FA MDS and AML which leads to the inactivation of this gene.^{22,25,26,46-48} The recurrent involvement of *RUNX1* in FA MDS/AML is of high interest because this is the first clear implication of a known oncogene in the multistep bone marrow progression in these patients, opening roads to figure out the oncogenic cellular pathways in FA. In addition, we found rare mutations of common oncogenes of non-FA MDS/AML, namely *NRAS* mutation, *FLT3-ITD* and *MLL-PTD*, whereas no mutations of *TET2*, *CBL*, *TP53*, *NPM1* and *CEBP α* were found in this cohort (Figure 4 and Supplemental Table S1). We also observed unique cases with abnormalities of the *PRDM16/MEL* or *ERG* oncogenes, as occasionally reported in non-FA patients.^{27-29,47,48} Combinations of the recurrent chromosomal or molecular abnormalities were frequent, no abnormality being clearly mutually exclusive of the others (Figure 4 and Supplemental Table S1). Collectively, these data suggest avenues of multi-step oncogenesis in the bone marrow progression of FA, in which 1q+ would be a central, possibly initiating event, that can be found in the aplastic form of the disease, and 3q+, -7/7q, and *RUNX1* abnormalities amongst other would lead to high grade MDS or AML. In this figure, 1q+ might clonally rescue the bone marrow failure of FA, partially or completely, but would not definitely protect against progression to MDS and leukemia. Serial studies of clonal evolution in humans or xenografts are needed to precise the order of the chromosomal/genomic events, and gene expression profiling should help to analyze their target genes and related cellular advantage in an aplastic bone marrow and during transformation.⁴⁹ Interestingly, we found that the *TP53* gene at 17p was not deleted or mutated in FA (except in a unique case with 5q-) (Figures 1 and 4), which was somewhat unexpected considering frequent mutation in non-FA patients with complex karyotype MDS/AML.⁵⁰ This suggests that in the specific FA background (constitutional genomic instability and excess of cell death), mechanisms other than *TP53* genomic mutation/deletion are likely to be more proficient. It is tempting to speculate that 1q+ or 3q+ might be implicated.

MDS/AML is a frequent and severe occurrence in FA. It is therefore important to follow up the patients by regular bone marrow examination with expert morphological and karyotype

analyses, in order to detect transformation before the onset of a high grade MDS/AML.^{1,2,5,7} In two patients with “normal” bone marrow in the present study, conventional karyotype appeared to be more sensitive than CGH/SNP-arrays for the detection of discreet 1q+ sub-clones, due to possibility to observe individual cells and probably to an advantage for mitosis in culture (Supplemental Table S1). Systematic interphasic FISH screening using probes for chromosome 7q, 3q (for instance a probe *EVI1*), and break-apart *RUNX1*, might increase the sensitivity of early detection of transformed cells. Importantly, we experienced several MDS/AML cases with a normal karyotype but cryptic chromosomal or genomic abnormalities detected by FISH and/or array analysis (Supplemental Table S1). Therefore, we now implement the cytogenetic analysis using high density DNA-arrays and *RUNX1* FISH when MDS or leukemia is diagnosed in FA patients. This approach is also useful to a more precise characterization of the abnormal karyotypes and frequently highlights meaningful chromosomal/molecular lesions (Figures 1-4; Supplemental Table S1 and Figure S2). Of note, if a SNP-array analysis is performed to search for somatic UPD regions (CN-LOH), paired BM and constitutive DNAs (like skin fibroblast DNA) analysis allows to rule out regions of constitutive homozygosity due to a degree of consanguinity (Figure 2). Finally and as also suggested by others,⁸ when 1q+ and/or 3q+ are found in a child or young adult with a *de novo* MDS or secondary-type AML, we carefully investigate an underlying diagnosis of FA, considering that physical signs of FA can be absent or very discreet and that the therapeutic consequences would be major.

In conclusion, bone marrow progression in FA is associated with a complex pattern of recurrent chromosomal abnormalities, some commonly found in non-FA MDS and secondary AML like -7/7q or *RUNX1* abnormalities, and some others specific from FA like 1q+ and 3q+. A careful analysis by karyotype, FISH, and in case of MDS/AML by high-density DNA arrays, allows a precise description of these clonal abnormalities. The prognostic value of the various chromosomal/genomic abnormalities will have to be carefully evaluated on the long term in large cohorts of FA patients with respect to the therapeutic options and clinical benefits. For instance, a sole clonal abnormality like 1q+ can be present in “normal” or non-MDS hypoplastic BM cells and, in our experience, it may persist for years without progression into MDS or AML (T.L., G.S. and J.S., unpublished data). Finally, the comprehensive collection of chromosomal/genomic data of FA patients constitutes a basis to figure out the step-wise hematopoietic clonal evolution and oncogenesis in a background of genomic instability, excess of cell death and bone marrow failure, with probable relevance non only in FA but also for non-FA patients with aplastic anemia or MDS/AML.

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Authorship and Disclosures

S.Q., arrays experiments, bioinformatic analysis and writing of the paper; W.C., cytogenetic analysis and writing of the paper, R.C., M.P.P., N.V., J.L., biological experiments; C.P., R.P., V.R., J.H.D., P.S., M.M., G.M., A.B., F.S., E.G., T.L. patient clinical care/follow up; C.D.E. and D.S.L., constitutional *FANC* mutation analysis; O.N. and C.P., oncogene mutation analysis; G.S., patient clinical care/follow up and study design; J.S., study design, biological and bioinformatic analyses and writing of the paper.

The authors reported no relevant conflicts of interest.

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Table 1. Clinical features in the 57 FA patients

Age	n
≤18 (range, 4 to 18)	29
>18 (range, 19 to 56)	28
Gender	
Female	31
Male	26
Consanguinity*	
Yes	15
No	38
Unknown	4
Constitutional <i>FANC</i> genes mutations	
<i>FANCA</i>	48
<i>FANCG</i>	1
<i>FANCF</i>	1
<i>FANCD2</i>	1
<i>FANCD1/BRCA2</i>	1
<i>FANCI/BACH1/BRIP1</i>	1
Unknown	4
Bone marrow morphology	
Normal [†]	8
Medullary hypoplasia / Aplastic Anemia [†]	20
Medullary hypoplasia	12
Aplastic Anemia	8
Myelodysplasia	18
RT	1
RARS	1
RCUD	2
RCMD	8
RAEB-1	4
RAEB-2	1
MDS-U	1
Acute Myeloid Leukemia	11
Karyotype^{††}	
Abnormal	29
Normal	24
Mitosis failure	4

* Consanguinity was defined according to the familial history and/or homozygous *FANC* gene mutation or deletion (when known); frequent homozygosity regions were found in the fibroblasts of these patients when tested by SNP-array (n=11).

[†] A moderate dyserythropoiesis is usual in bone marrow of FA patients and was not considered as a criteria for MDS.

^{††} At inclusion in the study.

RT, refractory thrombocytopenia; RARS, refractory anemia with ringed sideroblasts; RCUD, Refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB, Refractory anemia with excess of Blast; RAEB-1, 5-9% blasts; RAEB-2, 10-19% blasts; MDS-U, MDS-unclassifiable, accordingly to the World Health Organization classification 2008.

Table 2. Chromosomal and genomic abnormalities according to bone marrow stage

Chromosomal and genomic abnormalities	All pts with abn (n=35)	Normal BM* (n=8 pts)	MH / AA (n=20 pts)	MDS (n=18 pts)	AML (n=11 pts)	Significance NL* and MH/AA vs MDS and AML
Karyotype						
Abnormal	29 (82.8%)	3 (37.5%)*	4 (20.0%)	13 (72.2%)	9 (81.8%)	$P < 10^{-4}$
Complex (≥ 3)	9 (25.7%)	-	-	5 (27.8%)	4 (36.4%)	$P < 10^{-2}$
Array-CGH-SNP						
Abnormal	32 (91.4%)	1 (12.5%)*	5 (25.0%)	15 (83.4%)	11 (100%)	$P < 10^{-6}$
Recurrent abnormality						
1q+	19 (54.3%)	3 (37.5%)*	3 (15.0%)	7 (38.9%)	6 (54.5%)	NS
3q+	12 (34.3%)	-	-	7 (38.9%)	5 (45.5%)	$P < 10^{-3}$
21q/RUNX1	6 (17.2%)	-	-	2 (11.1%)	4 (36.4%)	$P < 0.05$
-7/7q-	5 (14.3%)	-	-	1 (5.6%)	4 (36.4%)	$P = 0.05$
11q-	5 (14.3%)	-	1 (5.0%)	3 (16.7%)	1 (9.1%)	NS
20q-	4 (11.4%)	-	2 (10.0%)	1 (5.6%)	1 (9.1%)	NS
5q-	3 (8.6%)	-	-	3 (16.7%)	-	NS
9p+	3 (8.6%)	-	-	2 (11.1%)	1 (9.1%)	NS
+8	2 (5.7%)	-	-	1 (5.6%)	1 (9.1%)	NS
Oncogene mutations ¶						
Yes	3 (8.6%)	-	-	-	3 (27.3%)	NS
Total pts with abnormalities	35 (100.0%)	3 (37.5%)*	5 (25.0%)	16 (88.9%)	11 (100.0%)	$P < 10^{-6}$
N. of abn per pt, for all pts (mean)	2.3	0.75	0.6	3.1	4.9	$P < 10^{-6}$
N. of abn per pt, in pts with abn (mean)	3.2	2.0	2.4	3.5	4.9	NS

* Inclusions of patients with normal bone marrow were enriched with patients who had abnormal karyotype and/or aged > 18 yo.

¶ Data observed from 25 patients; the mutation screen included TET2, CBL, NRAS, TP53, RUNX1, CEBP α , FLT3-ITD, FLT3-TKD, NPM1 and MLL-PTD.

NL, Normal bone marrow; MH, medullary hypoplasia; AA, aplastic anemia; MDS, Myelodysplastic syndrome; AML, acute myeloid leukemia.

** Fischer's exact test.

FIGURE LEGENDS

Figure 1. A recurrent pattern of somatic copy-number abnormalities and uniparental disomy (UPD) in FA bone marrow cells. All acquired copy-number and UPD regions found in the bone marrow cells of 57 individuals with FA are shown. Green lines represent deletions, red lines represent gains, and blue lines represent regions of UPD (somatic copy-neutral loss of heterozygosity). Constitutional copy-neutral homozygosity regions are not shown. The *RUNX1* gene location at 21q22 is shown.

Figure 2. SNP-array analysis of paired bone marrow and fibroblast DNAs distinguishes between constitutional homozygosity and acquired uniparental disomy. (A) Large chromosomal regions of constitutive homozygosity were detected by SNP-array in the DNAs of a male FA patients with a degree of consanguinity (patient EGF067, top panel), but not in a male heterozygote composite FA patient (patient EGF074, bottom panel). Homozygosity regions were detected by comparison with a HapMap reference set; fibroblast and bone marrow DNA analyses are shown on the left and on the right of each chromosome ideogram, respectively. The location of the *FANCA* gene at 16q24 is shown. Chromosome 13, which harbors both a constitutive homozygosity region in fibroblast (left) and a larger acquired UPD in the bone marrow only (right), is boxed. **(B)** Enlargement of chromosome 13 data from patient EGF067. Heterozygous (AB) and homozygous (AA and BB) calls and copy number data are indicated. The short constitutive copy-neutral region of homozygosity in fibroblasts (left) and the larger acquired UPD (copy-neutral loss of heterozygosity) in bone marrow (right) are boxed. Analysis was performed from Affymetrix SNP-array data with the Partek software.

Figure 3. *RUNX1/AML1* gene lesions in MDS/AML of several FA patients. (A) Overview of the chromosomal and molecular lesions detected on the *RUNX1* gene locus at 21q22 in the bone marrow cells of 6 FA patients with MDS/AML. Top panel, the genomic position of the *RUNX1* exons and of the FISH probes are shown; bottom panel, deletions and gains are represented in green and red, respectively. In patient EGF111, two unbalanced translocations involved chromosome 21, the most telomeric interrupting *RUNX1* as indicated; a balanced translocation detected in a sixth patient (EGF201) using the *RUNX1* flanking probes is not shown. **(B)** A 7bp insertion in *RUNX1* was found in the EGF171 patient in addition to the large deletion shown in A (blast cells 70%). **(C)** Break apart and duplication of the 5' telomeric *RUNX1* probe (green) in patient EGF036 with a t(1;21)(p36;q22). Original objective lens's magnification x63. A fusion *PRDM16-RUNX1* was confirmed by combinations of *RUNX1* and *PRDM16* probes (not shown).

Figure 4. Distribution of the chromosomal and molecular lesions in FA patients.

Somatic copy-number gains, losses and UPD, are color-encoded and plotted vertically in chromosomal order for each patient. Samples were clustered horizontally according to the progression stage groups in the bone marrow, and then by visual inspection to cluster the abnormalities. The recurrent abnormalities and a selection of relevant genes is indicated on the right part of the Figure. 1q+ and 3q+ are specific from FA and are indicated in red, the other abnormalities can be found in non-FA MDS/AML and are indicated in black. Oncogenes and tumor suppressor genes mutation screen data are shown on the lowest panel of the Figure, the dark grey background and brown boxes indicating which samples were screen and presence of mutations, respectively.

Figure 1

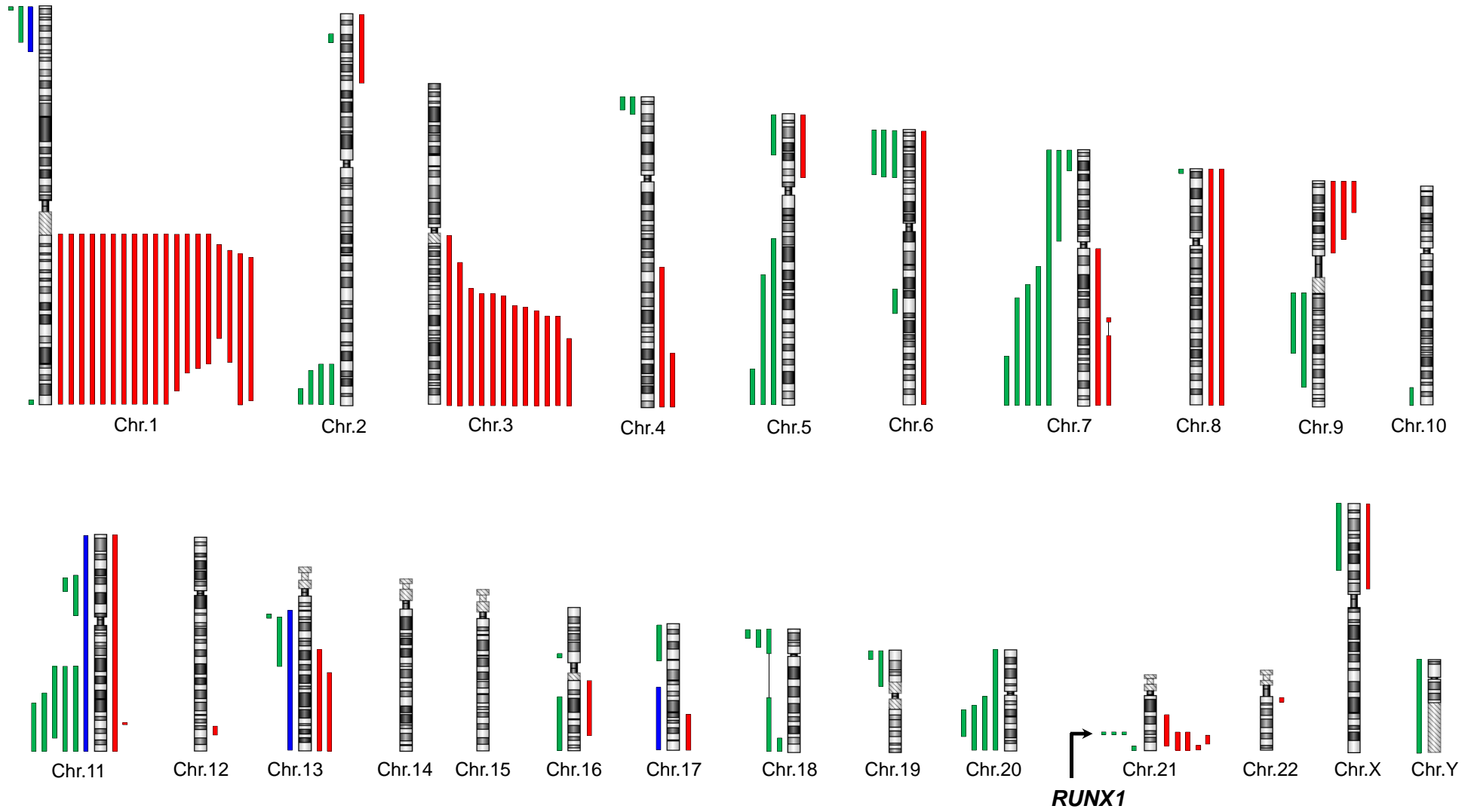
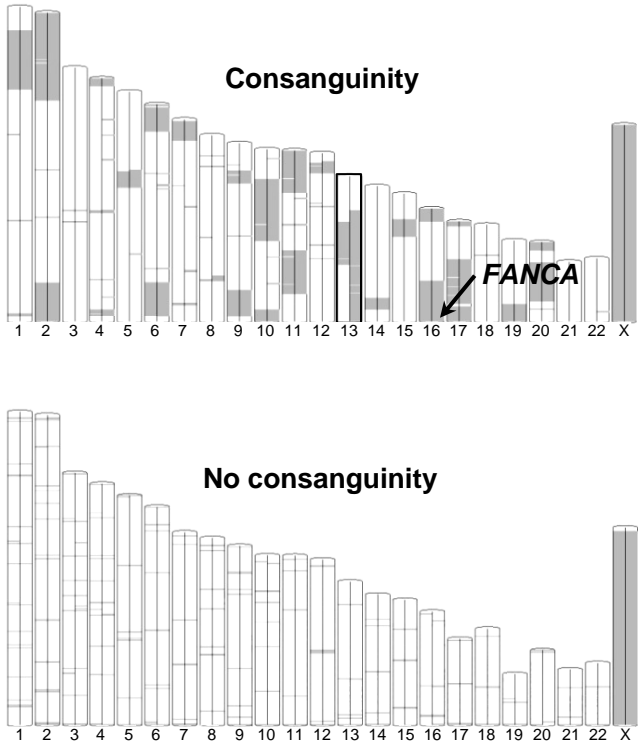


Figure 2

A



B

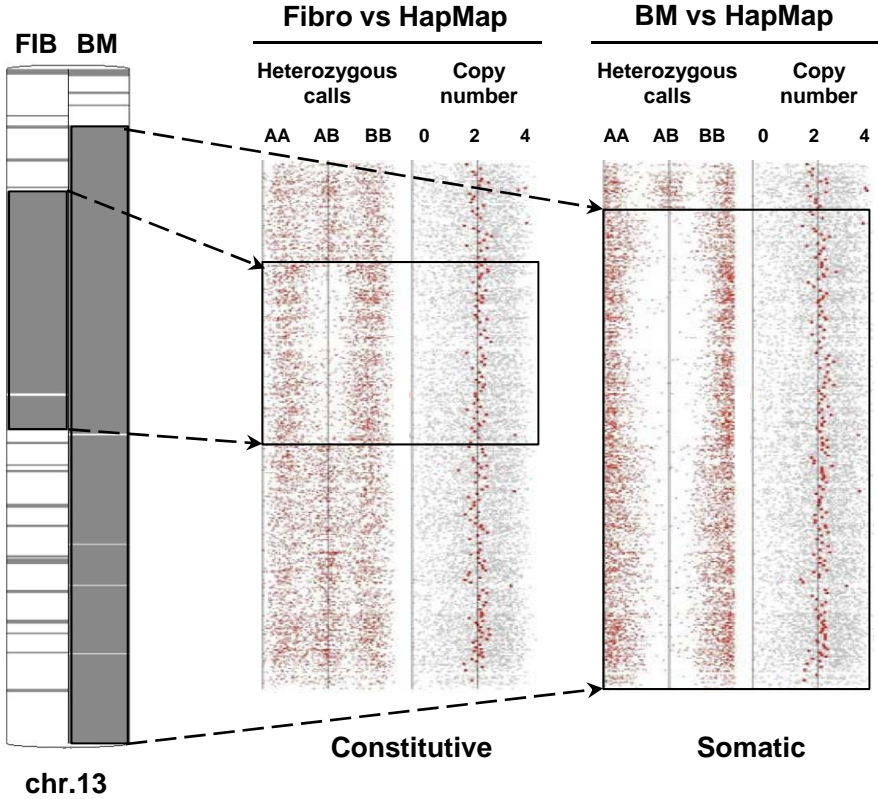
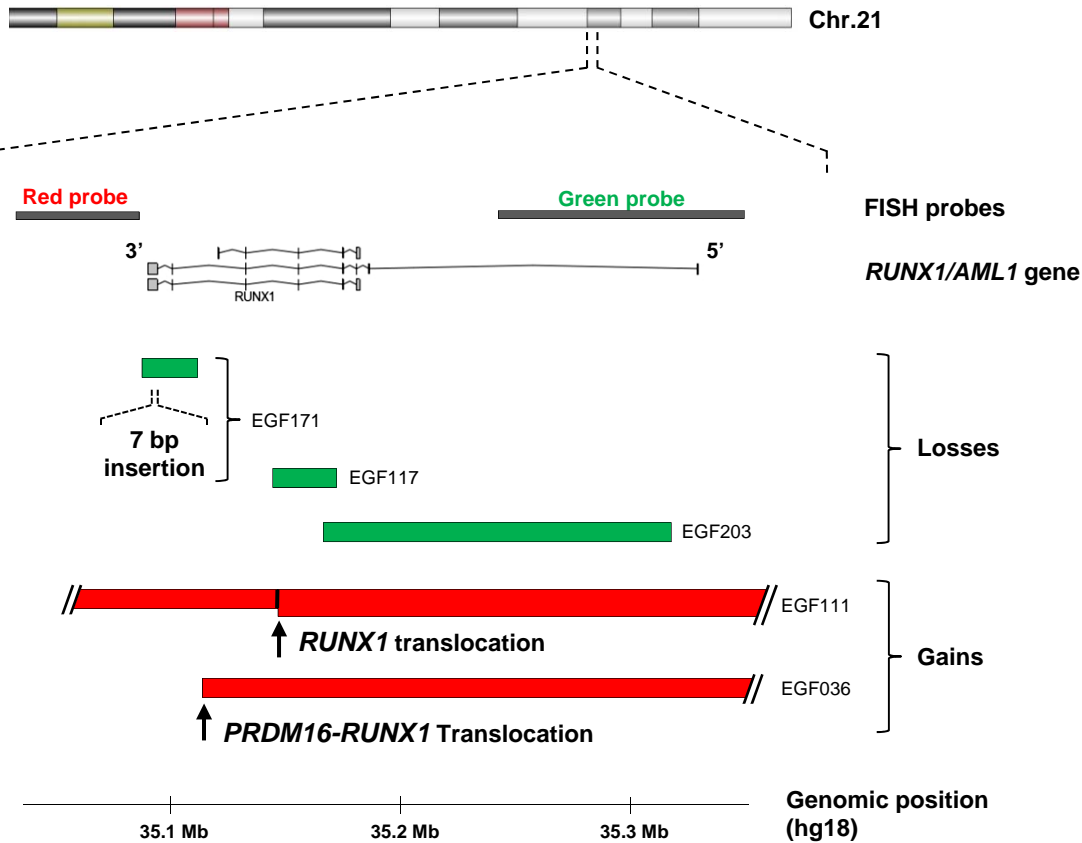
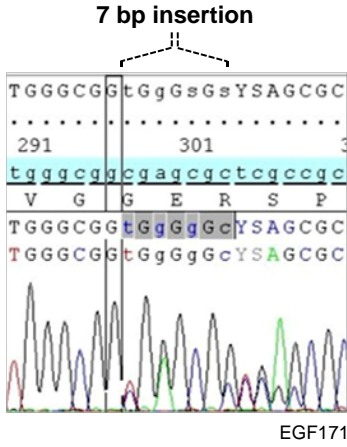


Figure 3

A



B



C

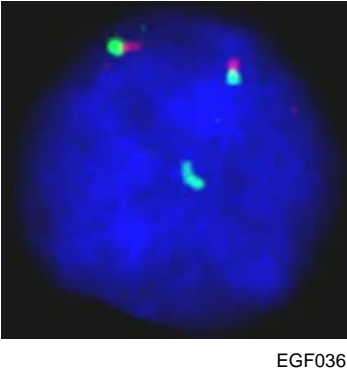
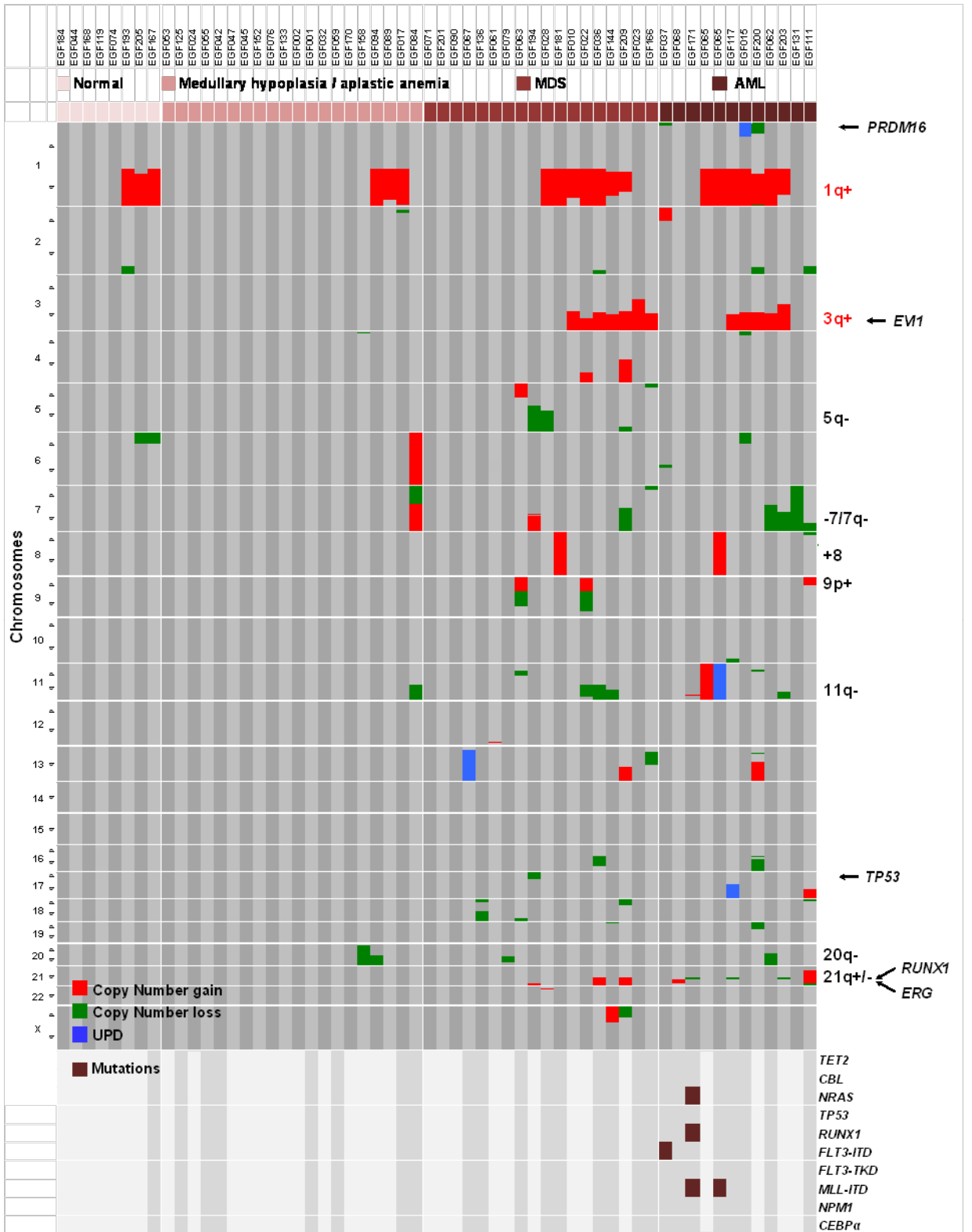


Figure 4





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Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic *RUNX1/AML1* lesions

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