Mutations of FLT3 and JAK2 promote inter chromosomal homologous recombination and the potential for copy neutral loss of heterozygosity (CN-LOH) in acute myeloid leukemia

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Abstract

Acquired copy neutral loss of heterozygosity (CN-LOH) is a frequent occurrence in myeloid malignancies and is often associated with resistance to standard therapeutic modalities and poor survival. Here, we show that mutated FLT3 and JAK2 constitutive signalling confers inter (between chromosome) homologous recombination (iHR), a precedent for CN-LOH. Using a targeted recombination assay, we determined significant iHR activity in internal tandem duplication FLT3 (FLT3-ITD) and JAK2V617F mutated cells. Moreover, sister chromatid exchanges (SCE) a surrogate measure of iHR was significantly elevated in primary FLT3-ITD normal karyotype acute myeloid leukaemia (NK-AML) compared to wild type (WT) FLT3 NK-AML. Homologous recombination (HR) is harmonised to S phase of the cell cycle to repair broken chromatids and prevent iHR. We demonstrated increased HR activity in G_0 arrested primary FLT3-ITD NK-AML in contrast to WT FLT3 NK-AML. Lastly, using a thymidine kinase (TK) gene mutation assay, mutated FLT3-ITD transfected cells demonstrated a significantly increased mutation frequency compared to WT transfection and that TK resistance was significantly associated with CN-LOH at the TK locus. Strikingly, treatment of FLT3-ITD and JAK2V617F mutant cells with the anti-oxidant, N-acetyl cysteine diminished reactive oxygen species (ROS) restoring iHR and HR levels observed for WT. These findings provide a proof of principle that mutated FLT3-ITD and JAK2 constitutive kinase activity augments ROS production and HR, shifting the cellular milieu towards illegitimate recombination events such as iHR and CN-LOH. Therapeutic reduction of ROS thus may prevent leukemic progression and relapse in myeloid malignancies.

Introduction

The majority of patients with adult acute myeloid leukemia (AML) that present with a normal karyotype (NK-AML) are grouped together in the "intermediate" risk cytogenetic category and constitute 40-45% of all adult AML patients. Mutations in the FMS-like tyrosine kinase 3 (FLT3) receptor whether it is internal tandem duplication (ITD) of its juxtamembrane domain or point mutations in its kinase domain are the most frequent mutations in NK-AML[1]. Constitutive activation of the JAK2 signalling pathway occurs in most myeloproliferative neoplasms (MPN) such as Polycythaemia Vera as well as in a significant proportion of other myeloid malignancies [2]. Both FLT3-ITD and JAK2 mutations drive uncontrolled hematopoietic cell expansion. FLT3-ITD mutation in NK-AML predicts a more aggressive disease associated with resistance to therapy and poor survival. However, the prognosis for AML patients that has lost the WT FLT3 allele and acquired the mutated FLT3-ITD is worse compared to patients maintaining WT FLT3. Loss of heterozygosity through duplication of genomic material on maternal or paternal chromosomes known as copy neutral LOH (CN-LOH), is a chromosomal change initially described in AML by Raghavan *et al* [3]. Since the discovery of CN-LOH (sometimes referred to as acquired uniparental disomy (UPD) in AML, recurrent regions of CN-LOH associated with homozygous mutations have been identified in AML [4]. In addition to the CN-LOH of FLT3-ITD at 13q, CN-LOH has been demonstrated at 4q and 9p in the presence of homozygous mutations of TET2 and JAK2 respectively, doubling the oncogenic gene burden [5]. Identification of multiple regions of LOH associated with mutation, points to a common mechanism of homozygosity in myeloid malignancies that is required for leukemic progression.

It is now generally accepted that acquired CN-LOH is the result of a homologous recombination (HR) event [6]. Mitotic HR is evoked in response to the repair of a double strand break (DSB) in DNA. Genomic integrity is maintained in the cell by specific DNA repair mechanisms such as HR and non-homologous end-joining (NHEJ). Nonetheless, defects in DNA repair can result in the inappropriate or abnormal repair of DNA damage resulting in propensity to malignant change. We have reported previously, that primary AML cells have increased NHEJ repair activity and

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accompanying repair infidelity. Furthermore, constitutively activated N-RAS results in an increase in reactive oxygen species (ROS) that damages DNA and evokes a DNA damage response (7-9). Our findings suggest that excessive DNA damage in AML may force normal DSB DNA repair components to process DSB aberrantly, resulting in chromosomal instability. It has been previously reported that constitutive FLT3 activation results in increased ROS production, a subsequent increase in DSB and inaccurate repair by the NHEJ pathway (10). The authors suggested that genomic instability derived from constitutive FLT3 signalling generates more mutations that facilitate leukemic progression. However, increased NHEJ cannot account for phenomena of CN-LOH.

To explore the relationship between constitutive FLT3 and JAK2 signalling and the propagation of CN-LOH we have utilised novel recombination assays that detect HR events in mutated FLT3 and JAK2 cells. We show that the FLT3-ITD and JAK2V617F mutation confer increased inter (between chromosomes) homologous recombination (iHR) activity compared to WT kinase. Moreover, iHR activity was reliant on the FLT3-ITD and JAK2V617F mutation dependent elevation of ROS. NK-AML patients with FLT3-ITD mutations also possessed significantly increased sister chromatid exchanges (SCE) (a surrogate for iHR activity), whilst a thymidine kinase (TK) reporter assay demonstrated FLT3-ITD activity preferentially increases resistance to TK through the creation of CN-LOH. Our results pinpoint a common pathway whereby constitutive FLT3 and JAK2 signalling confers iHR and the subsequent propagation of CN-LOH in normal karyotype myeloid malignancy.

Methods and Materials

Patients Twenty two patients with de-novo AML and normal karyotype by metaphase cytogenetics were selected. The median age was 53 years (8-78 years) and the median white blood cells count was 51.3 x 10^9/l. Primary material was obtained following written informed consent from patients prior to inclusion in study in accordance with the declaration of Helsinki by King's College Hospital Local Research Ethics Committee.

Drugs The FLT3 inhibitor, AC220 (Quizartinib) was purchased from Selleckchem and re-suspended in PBS. N-acetyl cysteine (NAC), Hydrogen peroxide (H_2O_2) and the RAD51 inhibitor, B02 were purchased from Sigma-Aldrich, Poole UK.

Plasmids and DNA repair substrates Human FLT3 and FLT3-ITD (18bp insertion) have been previously cloned into FUGW plasmid (kind gift from Feyruz Rassool, University of Maryland). The *BAMH*1-*ECOR*1 GFP fragment was removed from FUGW prior to cloning. Expression constructs, pMSCV-FLT3-WT and pMSCV-FLT3-ITD were described previously (11-12) and were kindly donated by Lars Rönnstrand, University of Malmo. PMSCVneo WT JAK2 and pMSCVneo JAKV617F (13) were kindly donated by Gary Reuther, University of South Florida. DNA repair substrate, DR-GFP and the *I-SCE*I expressing plasmid, pCBASCEI have been described previously by Maria Jasin's group (14-15) and were purchased via the Addgene repository (DR-GFP, Addgene plasmid 26475 and pCBASCEI, Addgene plasmid 26477). Creation of targeted repair substrates is provided in supplementary methodology.

Targeted Recombination assays To study targeted iHR, DNA repair substrates; PZD-DR1-Puro (10µg) and PZD-DR2-Blast (10µg) were simultaneously transfected (Nucleofection, Amaxa) into 2 x 10^6 cells using the CompoZr Zinc finger nuclease (ZFN) targeted integration kit (Sigma) according to the manufacturer's instructions. To analyse targeted HR, PZD-DR1/DR2-Puro (20µg) was transfected into 2 x 10^6 cells also using CompoZr ZFN. Two days after transfection, cells were antibiotic selected and divided into 24 well plates to isolate

correctly targeted clones. After 5 days, cells were collected for FISH analysis and PCR identification of integration at the AAVS locus. Only correctly targeted clones with PZD-DR1-Puro and PZD-DR2-Blast were selected and expanded. To correctly targeted HEK293 clones, 2µg of WT, FLT3-ITD, JAK2V617F or empty expression vector were transfected into 2×10^6 cells. Antibiotic selection (200µg/ml Zeocin for FUGW-FLT3-ITD, 500µg/ml G418 for pMSCVneo-JAK2V617F) were added 24h after transfection in fresh media. Relative expression of FLT3 and JAK2 was determined by quantitative PCR and western blotting 3 days after antibiotic selection. 10µl of the *I-SCE*I expression viral supernatant was added to PZD-DR1-Puro and PZD-DR2-Blast targeted cells and cultured for a further 5 days before FACS analysis. For simultaneous drug additions, 10-50nM AC220 or 5mM N-acetyl cysteine (NAC) were added for 24h prior to addition of *I-SCE*I. To account for variation in transfection efficiencies between test cells, the targeted recombination % for each test was made relative to the transfection of PZD-GFP-Puro GFP expression.

Cell Lines The leukemic cell lines, MOLM-13, U937, and the embryonic kidney cell line, HEK293 were obtained from the DSMZ. The lymphoblastic cell line, TK6 was obtained from the ATCC. These cell lines were purchased within the last three years, propagated, expanded, and frozen immediately into numerous aliquots after arrival. The cells revived from the frozen stock were used within 10–15 passages and not exceeding a period of 6 months. The DSMZ and ATCC use morphological, cytogenetic and DNA profile analysis such as STR fragment analysis for characterization and authentication of cell lines. Prior to the study start, all cell lines were subjected to mutation analysis using Roche 454 parallel sequencing for authenticity compared to the genotype characterised by the DSMZ and ATCC.

PCR confirmations To confirm specificity of targeted integration 5 days after transfection, DNA was prepared from transfected cells (Qiagen). Site specific PCR was carried using the CompZr AAVS forward primer and a reverse primer specific to PZD-DR1-Puro and PZD-DR1/DR2-puro, (5'-CTT GTA CAG CTC GTC CAT GC) and to PZD-GFP-puro and PZD-

DR2-Blast, (5'-CAG TGC AGG AAA AGT GGC ACT). PCR was carried out according to the CompZr integration PCR protocol using Roche Expand High Fidelity PLUS polymerase. To confirm the recombination at the I-*SCE*I site, recombined GFP positive cells were FACS sorted (FACSaria, Beckman Coulter) into 96 well plates to collect GFP positive cells. Cells were used for hot start PCR across the DR1 region using forward primer: CTG CTA ACC ATG TTC ATG CC and reverse primer: AAG TCG TGC TGC TTC ATG TG. The PCR product was digested with 10U of *I-SCEI* or 10U *BCG*1. GFP positive PCR products attributable to HR should be *I-SCE*I un-digestible and *BCG*1 digestible. Products were loaded onto gels and stained with ethidium bromide, and the ethidium signals for the enzyme-resistant and enzyme-cleaved band were quantified by using Adobe Photoshop software.

Fluorescent hybridisation (FISH) PZD targeting vectors were fluorescently tagged for FISH studies using the Fasttag labelling kit (Vector, Burlingame CA). 10μg of vector and was denatured at 95°C for 30min in the presence of Fasttag reagent. Vectors were subsequently coupled with either fluorescein or Texas Red maleimide. Labelled vector DNA was determined to be between 100-400bp by agarose gel electrophoresis. Metaphase spread chromosomes were prepared as stated for the analysis of sister chromatid exchanges. After the slides were dried overnight, the slides were spotted with 200μl ethanol and placed on a hotplate for 20min. The slides were then cooled and immersed in 2% SSC for 20min before dehydration in an ethanol series (70%, 85%, and 100%). Fluorescently labelled vector was diluted to 1ng/μl in hybridisation buffer (60% deionized formamide, 2 X SSC, 10% dextran sulfate, 50mM sodium phosphate pH7). The fluorescently labelled vector was denatured at 75°C for 5 min prior to addition to the slide and sealing with rubber cement. The slide was then denatured on a hotplate for 4min at 75°C. Slides were then placed in humid boxes overnight at 37°C. After hybridisation, slides were blocked in blocking solution (5% non-fat dry milk plus 0.1% tween 20 in 4 X SSC) for 60min at 37°C. The slides were then washed in blocking solution and subsequently incubated in biotinylated anti-fluorescein or anti-Texas

Red (10μg/ml) in blocking solution for 30min at RT. Slides were washed again 3 times in blocking solution and then incubated in fluorescein or Texas Red Avidin solution (10μg/ml) for 30min at RT. Slides were washed in 4 x SSC + 0.1% tween 20 (2 x 5min) before staining with DAPI (100μg/ml) for 5 min and mounting in vectashield. SureFish 19q 13.11 CEBPA probe (Agilent) was used as a control for vector targeting the AAVS1 locus. Hybridisation of probe was as manufacturer's instructions. Slides were visualized using a Leica epi-fluorescent microscope, and images were captured using a CCD camera and Leica LAS AF software. Two slides were prepared for each experiment.

TK6 assay The lymphoblastoid cell line, TK6 was cultured in CHAT media (10μM deoxycytidine, 200μM hypoxanthine, 0.1μM aminopterin and 17.5μM of thymidine) for 3 days to reduce the background mutant fraction. TK6 were then transfected with empty vectors, FUGW and pMSCV or FUGW WT-FLT3, FUGW FLT3-ITD selected in 200μg/ml Zeocin or pMSCV-WT-FLT3 and pMSCV-FLT3-ITD selected in 1μg/ml puromycin for 24h and then cultured for 5 days. To determine the mutation rate, 2×10^5 cells was seeded into ClonaCell™-TCS Medium (Stem Cell Technologies, France) with and without 2μg/ml trifluorothymidine (TFT) and incubated for a further 10 days. The mutation frequency was calculated by dividing the number of colonies on TFT containing plates by the number of colonies on the plates without TFT. Triplicate plates for all tests were produced. The mutation rate was determined by seeding 2×10^5 cells into 25 wells of media containing TFT and determining the fraction of wells without TFT resistance. The Mutation Rate was calculated using the Fluctuation test originally described by Luria and Delbrück (17) using the formula.

$$\text{Mutation Rate} = -\ln x \; \frac{h}{n}$$

(ln - natural log, h – Number of wells without resistance/number of wells, n – number of cells)

Additional methods can be found in supplementary methodology

Results

Development of a Zinc Finger Nuclease (ZFN) targeted assay for the determination of iHR

We firstly developed a novel reporter assay targeted to chromosome 19q using CompoZr™ Zinc finger nuclease (ZFN) to detect iHR (Figure 1A-D). The assay, based on the DR-GFP reporter developed by Jasin *et al*, relies on the sequential targeting of two overlapping, but disrupted GFP repeat sequences to separate alleles at the adeno associated virus integration site 1 (AAVS1) locus (14-16). The two GFP repeat sequences, 5'GFP and 3'GFP were individually cloned into the targeting vectors, PZDonor-AAVS1 Puromycin (Figure 1 A,C and PZDonor (Figure 1 B,D) respectively to create PZD-DR1-Puro and PZD-DR2-Blast respectively. PZD-DR1-Puro and PZD-DR2-Blast can be sequentially targeted to AAVS1 on separate 19q alleles with concomitant transfection with AAVS1 specific ZFN. iHR and subsequent gene conversion between the disrupted GFP sequences would result in GFP expression. To test the specificity of ZFN targeting, we initially cloned the full length intact GFP into PZDonor-AAVS puromycin and transfected this vector with AAVS1 specific ZFN into HEK293 cells. Supp. Figure 1A-B shows correct integration of PZD-GFP-Puro into the AAVS1 locus using locus specific primers and FISH. Integration efficiency before antibiotic selection was between 10-15% for cell lines (data not shown). Without ZFN transfection, there was no AAVS1 integration and no puromycin resistant cells after selection. Targeted integration of PZD-DR1-Puro and PZD-DR2-Blast to the AAVS1 locus was performed in the FLT3-ITD mutated cell line, MOLM-13 (known hereon as MOLM-13PZD), the WT FLT3 U937 (known hereon as U937PZD) and HEK293 (known hereon as HEK293PZD). Targeted integration was confirmed using locus specific PCR and FISH (Figure 1 E-G). Clones with both the correct integration of PZD-DR1-Puro and PZD-DR2-Blast to separate AAVS1 loci were then expanded in culture.

FLT3-ITD cells demonstrate iHR

FLT3-ITD expressing cells were tested for iHR events using the targeted iHR reporter assay. MOLM-13PZD and FLT3-ITD transfected HEK293PZD cells demonstrated reproducible GFP positive cells in three separate experiments (iHR events, 0.15% and 0.25% respectively compared to U937PZD, WT-FLT3 and empty vector transfected HEK293PZD cells (0%, $p < 0.05$, n=3) (Figure 1H, Table I). Induction of iHR in FLT3-ITD transfected HEK293PZD corresponded to a 4-fold increase in RAD51 expression after normalisation for FLT3 overexpression (Supp. Figure 2A). Pre-treatment of MOLM-13PZD and FLT3-ITD transfected HEK293PZD cells with the FLT3 inhibitor, AC220 down-regulated RAD51 expression with the concomitant abolition of iHR events (Figure 1H, Supp. Figure 2 B-C, Table I). Furthermore, iHR were also eliminated with pre-treatment with the anti-oxidant, N-acetyl cysteine (NAC) (Table I, Supp. Figure 2B). Interestingly, iHR events were significantly dependent on both HR activity and ROS as pre-treatment of MOLM-13PZD and FLT3-ITD transfected HEK293PZD cells with the RAD51 inhibitor, B02 abolished iHR events, but addition of the pro-oxidant, hydrogen peroxide (H_2O_2) increased iHR events (Table I, Supp. Figure 2B). To verify that GFP positive cells in this assay were the result of iHR and not false positives, hot start PCR using primers designed at the DR1 locus was carried out on FACS sorted GFP positive cells. PCR products from GFP positive cells could only be digested by *BCG*1 and not *I-SCE*I confirming the presence of iHR dependent GFP positive cells (Figure 1 I-J). GFP positive cells were also verified using fluorescence microscopy (Supp. Figure 2D).

FLT3-ITD mutation increases HR events in primary NK-AML

Increased HR has been shown previously in FLT3-ITD AML (17). To investigate further the presence of elevated HR as the catalyst for iHR in primary FLT3 mutated AML, we measured targeted single locus HR in a cohort of NK-AML. Primary NK-AML (n=22) were

initially characterised by SNP-A and DNA sequencing (Supp. Table I). 15/22 primary NK-AML had FLT3-ITD mutations with a clone size ≥ 25%. 7/22 primary NK-AML were WT for FLT3, but had nucleophosmin (NPM1) and DNA methyltransferase 3 (DNMT3a) mutations. We developed a novel HR reporter assay, using the HR substrate, PZD-DR1/DR2-Puro that possesses two disrupted, but overlapping GFP sequences targeted to AAVS1 (Supp. Figure 3A, supplementary information). HR between the two sequences would result in GFP expression. PZD-DR1/DR2-Puro was transfected into primary FLT3-ITD with CompoZr™ ZFN. Supp. Figure 3B-D shows correct integration of PZD-DR1/DR2-Puro into the AAVS1 locus using locus specific primers and FISH. Clones with the correct integration of PZD-DR1/DR2-Puro AAVS1 were further transfected with an *I-SCE*I expression viral supernatant to introduce a double strand break in the integrated PZD-DR1/DR2-Puro and induce recombination. PZD-DR1/DR2-Puro/*I-SCE*I transfected primary FLT3-ITD (n=4) demonstrated significantly increased HR activity compared to primary WT FLT3 (n=2), (1.2 vs 0.25%, $p<0.01$) (Figure 2A, Supp. Table II). The increased HR activity was not attributable to elevated I-*SCE*I expression in primary FLT3-ITD after lentiviral transduction, but minimal cytotoxicity was noted at high I-*SCE*I concentrations in all primary cells that did not affect recombination rates (Supp. Figure 4A). All measurements of PZD-DR1/DR2-Puro HR events were made relative to transfection of PZD-GFP-Puro + ZFN to correct for variable transfection efficiencies in primary AML. Integration efficiency before antibiotic selection was between 5-15% for primary cells (Supp. Figure 4B). Pre-treatment of primary cells with AC220 or NAC significantly inhibited HR events (1.2 vs 0.1%, $p<0.01$)(Figure 2A, Supp. Table II). Moreover, PZD-DR1/DR2-Puro/*I-SCE*I transfected MOLM-13 and U937 + FLT3-ITD also exhibited increased targeted HR that was inhibited with AC220 or NAC (mean across all groups, 2.6 vs 0.35%, $p<0.01$) (Supp. Table III, Supp. Figure 4 C-D). Inhibition of HR in primary NK-AML correlated with the reduction in RAD51 expression with AC220 (Figure 2B). Expression of HR repair factors, MRE11 and CTiP were not affected by AC220, but the expression of cyclin dependent kinase inhibitor, p21 was abrogated as previously shown by Raderschall *et al (*18). FLT3-ITD expression also resulted in the phosphorylation

of both AKT and STAT5 in primary NK-AML and FLT3-ITD transfected HEK293 that was also abrogated upon AC220 pre-treatment (Figure 2 B-C). Mononuclear cells from this cohort of primary NK-AML, MOLM-13 and FLT3-ITD transfected U937 were also cultured and assessed for their ability to localise phospho-γH2AX and RAD51 as a measure of DSB DNA damage and HR activity respectively. Immunofluorescence studies showed that primary AML with FLT3-ITD, MOLM-13 and FLT3-ITD transfected U937 cells have increased mobilization of phospho-γH2AX and RAD51 compared to WT FLT3 primary AML cells and U937 transfected with WT FLT3 (Mean for all groups; RAD51, 21% vs 10.3%, $p<0.01$; phospho-γH2AX 17 vs 12.7%, $p<0.01$, n=3) (Figure 2 D-E, Supp. Figure 5 A-C). Pre-treatment of cells with NAC significantly inhibited both RAD51 foci formation (21 vs 14%, $p<0.01$) as well as phospho-γH2AX foci (17 vs 11.6%, $p<0.05$). Moreover, there was a direct correlation between the increased RAD51 and phospho-γH2AX foci and the augmentation of ROS for FLT3-ITD positive NK-AML (Figure 2 E-F, Supp. Figure 5 A-E).

Primary AML with FLT3-ITD mutations demonstrate increased sister chromatid exchanges (SCE)

Given the increased HR in mutated FLT3 cells, recombination or 'crossing over' is likely to occur between chromatids following DNA breakage. This phenomenon is known as sister chromatid exchanges (SCE). Inadvertent slippage of a broken chromatid could result in the use of its homologous chromosome for repair, supporting the utility of SCE analysis as a surrogate for iHR. To confirm the presence of elevated recombination in primary FLT3 mutated AML, we measured SCE in 22 NK-AML. FLT3-ITD primary AML, MOLM-13 and FLT3-ITD transfected U937 cells show a significant increase in SCE compared to WT FLT3 primary AML and WT FLT3 transfected U937, (mean across groups, 9.9 vs. 5.5 SCE per metaphase, $p<0.001$) (Figure 2 G-H, Supp. Fig. 6A-C, Supp. Table II and III). Moreover, treatment of FLT3-ITD primary AML, MOLM-13 and FLT3-ITD transfected U937 cells with

the AC220 or NAC restored SCE to levels observed for WT FLT3 (mean across groups AC220: 9.9 vs. 5.9 SCE per metaphase, p<0.05; NAC: 9.9 vs 6.7 SCE, per metaphase, p<0.05).

JAK2V617F mutation increases targeted iHR events

Constitutive activation of the JAK2 signalling pathway occurs in most myeloproliferative neoplasms (MPN) such as Polycythaemia Vera as well as in a significant proportion of other myeloid malignancies. JAK2 mutations such as V617F give rise to a proliferative phenotype that has also shown to induce increased ROS production (19). As JAK2V617F is associated with CN-LOH on chromosome 9p in MPN we determined whether JAK2 and FLT3 mutations share a common mechanism in initiating CN-LOH. The JAK2V617F mutated leukemic cell line, HEL and JAK2V617F transfected HEK293 demonstrated 4 fold increased RAD51 expression compared to U937 and WT JAK2 transfected HEK293 (Figure 3 A-B). JAK2V617F expression also resulted in the phosphorylation of both AKT and STAT5 in JAK2V617F transfected HEK293. Targeted integration of PZD-DR1-Puro and PZD-DR2-Blast to the AAVS1 locus was performed in the JAK2V617F mutated cell line, HEL (known hereon as HELPZD) and showed significant iHR events compared to U937PZD (0.2% vs 0%, p<0.05, n=3)(Figure 3C). Furthermore, we were able to abolish iHR events with NAC. HEK293PZD cells were transfected with JAK2V617F and also demonstrated reproducible GFP positive cells (iHR events, 0.3% compared to WT JAK2 and empty vector transfected HEK293PZD cells (0.3% vs 0%, p<0.05, n=3) (Table I). We also show that HEL and JAK2V617F transfected HEK293 cells demonstrate increased HR compared to HEK293 + WT JAK2 (2.6% vs 1%, p<0.05, n=3) and that ROS is significantly elevated in JAK2V617F cells (Figure 3 D-F).

Inappropriate RAD51 expression and HR activity in G_0 arrested FLT3-ITD cells.

RAD51 expression is tightly regulated in normal cells with the highest expression being at S/G_2 phases during and after DNA replication and its lowest expression at G_0/G_1 phases or in non-dividing cells (6). As FLT3-ITD mutation results in significantly overexpressed RAD51 we investigated whether there was inappropriate expression of RAD51 in FLT3-ITD mutated cells outside of S/G_2 that may result in recombination between homologous chromosomes rather than between identical chromatids after replication. Primary NK-AML and MOLM-13 cells were initially cultured in full growth media and then in the absence of foetal calf serum for 3 days. G_0 arrested primary FLT3-ITD NK-AML and G_0 arrested and FACS sorted MOLM-13 demonstrated increased RAD51 foci localisation and low, but consistent RAD51 expression compared to G_0 arrested primary WT FLT3 AML and G_0 arrested FACS sorted U937 cells (Figure 4 A-D, Supp. Fig. 7A-F). Strikingly, G_0 arrested WT FLT3 primary AML and G_0 arrested U937 cells only exhibited RAD51 expression 12h after culture stimulation with growth factors. Transfection of the HR substrate, PZD-DR1/DR2-Puro and subsequent induction of a DNA DSB by I-*SCE*I transfection showed that G_0 arrested FACS sorted MOLM-13 demonstrated low, but reproducible HR events compared to G_0 arrested FACS sorted U937 cells. Furthermore, G_0 MOLM-13 HR events were abolished with co-culture with AC220 or NAC (Figure 4G).

FLT3-ITD mutation promotes CN-LOH over hemizygosity at the thymidine kinase (TK) locus

The human lymphoblastic cell line, TK6 possesses heterozygous frameshift mutations in exon 4 and exon 7 of thymidine kinase (TK) (20). Loss of heterozygosity (LOH) at this locus renders resistance to trifluorothymidine (TFT). We exploited LOH at the TK locus to study the effects of mutant FLT3-ITD expression. TK6 cells were transfected with FLT3-ITD or WT FLT3 or empty vectors and subsequently cultured in TFT to allow the generation of TK mutations. Figure 5A and Supp. Table IV show that spontaneous TK mutation rate and

frequency were significantly elevated in FLT3-ITD transfected TK6 clones compared to WT FLT3 and empty vector transfected TK6 clones. We demonstrated this mutation phenotype using 2 different FLT3-ITD expressing vectors (FUGW and pMSCV). Strikingly, for both FLT3-ITD expressing vectors, mutant rate and frequency were significantly ameliorated with addition of AC220 or NAC. To determine the type of LOH if any at the TK locus, clones from FLT3-ITD, WT FLT3 and empty vector controls were used for fragment PCR analysis. More than 20 TK deficient clones were analysed per group. Figure 5B-C demonstrates that in FLT3-ITD transfected TK6 mutants, more than 50% of clones exhibited homozygous LOH at the TK locus in contrast to empty vector controls. The remaining 50% in FLT3-ITD transfected TK6 gave no LOH, whilst hemizygous LOH was not observed. Significantly, CN-LOH frequency at the TK locus was inhibited with concomitant addition of AC220 (55 vs 28%, p<0.01) and NAC (55 vs 26%, p<0.01). Microsatellite analysis of the q arm of chromosome 17 was also used to confirm CN-LOH in TK mutants. Nine microsatellites were chosen, 8 located to the q arm and one to the p arm. From 34 TK resistant colonies derived from FLT3-ITD transfected TK6 clones, we determined 3 sizes of CN-LOH all extending to the telomere (Figure 5D). FLT3-ITD transfected TK mutants demonstrating homozygous LOH at the TK locus establishes an enhanced propensity of such mutations to confer CN-LOH and can abrogated through inhibition of constitutive FLT3 activity or anti-oxidant treatment.

Discussion

Oncogenes such as FLT3 and JAK2 play key roles in hematopoietic stem cell proliferation, differentiation and survival (21-23). Mice that are heterozygous for the FLT3-ITD mutation develop a chronic myeloproliferative disease and must acquire other mutations or cellular changes to develop the full AML phenotype. Other molecular changes must also occur to allow the spontaneous loss of heterozygosity in these oncogenes that increases the severity of AML. Since our original report detailing CN-LOH in low risk myelodysplastic syndrome (24) there have been a plethora of reports documenting the prevalence of CN-LOH in a number of other hematological disorders (reviewed by O'Keefe *et al,* 4). Furthermore, we and others have associated microsatellite instability (MSI) as an instigator of CN-LOH (25-26). However, the aetiology of CN-LOH acquisition remains unknown. In this report, we provide comprehensive evidence of a common mechanism of CN-LOH in NK-AML where excessive ROS derived DSB DNA damage is preferentially repaired by mutant FLT3 or JAK2 dependent elevation in iHR and HR.

ROS has been previously shown to be elevated by mutant FLT3 (10), JAK2 (19) and BCR-ABL (27) resulting in DNA damage, genomic instability and leukemic progression. Indeed, we have previously demonstrated that a constitutively activated mutant N-RAS mouse model augments ROS production resulting in DSB DNA damage and subsequent up-regulation of NHEJ repair activity. Mice fed with the anti-oxidant, N acetyl cysteine (NAC) demonstrated a significant reduction in ROS, diminished DNA damage and NHEJ activity (9). We demonstrate here that mutant FLT3 and JAK2 confer augmented ROS species that induce DNA damage resulting in both mutations and chromosomal instability as observed in the TK assay that can be abolished with pre-treatment with anti-oxidants. Such genomic instability is the prerequisite for leukemic progression. Interestingly, Both STAT5 and AKT were both phosphorylated in FLT3-ITD and JAK2V617F cells. STAT5 dependent activation of NADPH oxidase in association with RAC1 results in increased ROS, whilst AKT activates FOXO3 to

abrogate catalase (inhibitor of ROS) (28). A contribution of both pathways is likely to provide the high levels of ROS to drive genomic instability and leukemic progression.

Repair of DSB requires the co-ordinated actions of both NHEJ and HR to insure genomic fidelity and negate chromosomal aberrations. NHEJ is the predominant mammalian pathway being active throughout the cell cycle, whilst HR is primarily active during S phase during DNA replication. Aberrant interplay between these repair activities has a profound effect on DNA repair and genomic fidelity. In accordance with other reports, mutant FLT3 and JAK2 demonstrated increased RAD51 expression resulting in exaggerated HR activity through the binding of phosphorylated STAT5 to the RAD51 promoter (29). It is notable, that mutant FLT3 and JAK2 are associated with CN-LOH at their respective loci resulting in a more aggressive phenotype. Similarly, mutant TP53 that is also associated with CN-LOH at 17p demonstrates increased RAD51 expression and HR activity (29). The elevated HR activity not only results in increased resistance to traditional chemotherapy, but could also drive the propagation of iHR. Here, we provide a model where FLT3-ITD and JAK2V617F dependent constitutive activity drives the propagation of CN-LOH that can be prevented with anti-oxidant treatment (Figure 5E). Mutated FLT3 or JAK2 drives the accumulation of ROS that damages DNA and is preferentially repaired by FLT3-ITD and JAK2V617F dependent augmentation of the HR repair pathway,that increases SCE and also the likelihood of increased iHR events. A specific iHR event at 13q for FLT3 or 9p for JAK2 would result in the doubling of mutant gene dosage and selection for malignant progression. However, the observed overactive HR may not by itself be sufficient to confer iHR. To this end, we also demonstrate inappropriate expression of HR activity in G_0 cells in primary FLT3-ITD cells. HR is tightly regulated to be active after DNA replication to repair broken chromatids. Breaks in one chromatid can be accurately repaired using its identical sister by HR. Prior to DNA replication in G_1, NHEJ repairs DSB, however, illegitimate HR expression in G_1 has the capacity to repair breaks between homologous chromosomes resulting in iHR events and CN-LOH. Further, we show that mutated FLT3 also confers increased expression of the

cyclin dependent kinase inhibitor; p21 in primary NK-AML; mediating G_1 arrest and slower progression to S phase increasing the likelihood for iHR.

The proposal that CN-LOH is not merely a stochastic event brought about by selection pressure of a leukemic clone, but a direct result of constitutional FLT3 and JAK2 activity has considerable relevance to the biology and therapy of myeloid malignancies. The disparate and independent activities of a single oncogene having the capacity to combine to create CN-LOH may be a common mechanism in these diseases. The identification of this mechanism of CN-LOH propagation will also open up further avenues of investigation. The TK assay revealed a bias in FLT3-ITD transfected cells to specific sites and sizes of CN-LOH. The uniformity of the CN-LOH suggests that common breakpoints at the TK locus were responsible for the CN-LOH. Similarly, CN-LOH at 13q may be a reflection of hotspots of recombination at the locus rather than simply a selection of a mutant FLT3 homozygous clone. Targeting integration of DNA repair substrates at CN-LOH rich loci could be utilised to determine preferential sites of recombination and their correlation to fragile sites. ROS induced DNA damage has been postulated to induce mutations to accelerate leukemic progression and relapse (9). The present study makes a valid case for the use of anti-oxidants in haematological malignancy given the observed ROS accumulation in CML, AML, MPN and CLL (30). Such treatments working alone or in combination with conventional therapies could prevent the accumulation of mutations and the acquisition of CN-LOH in NK-AML slowing the rate of disease progression. Furthermore, the side-effects of using high dose DNA damaging chemotherapy could be avoided if low doses can be combined with anti-oxidants. Our findings suggest that novel therapies targeting the generation of ROS would prove to be of great importance clinically for treatment of mutant FLT3 and JAK2 myeloid malignancies.

Acknowledgements

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Table I. Summary of iHR mediated events in mutant FLT3-ITD and JAK2V617F cells. Percentages are the result of replicate studies, (n=3)

Cell	Targeted iHR (Mean % ± SEM GFP cells)				
	Untreated	+ 25nM AC220	+ 5mM NAC	+ 25µM B02	+ 50µM H_2O_2
MOLM-13PZD	0.15 ± 0.05	0	0	0	0.25 ± 0.05
U937PZD	0	0	0	0	0
HEK293PZD + EV	0	0	0	0	0
HEK293PZD + WT FLT3	0	0	0	0	0
HEK293PZD + FLT3-ITD	0.25 ±0.05	0	0	0	0.4 ±0.05
HELPZD	0.2± 0.05	0	0	0	0.15
HEK293PZD + WT JAK2	0	0	0	0	0
HEK293PZD + JAK2V617F	0.3 ± 0.05	0	0	0	0.4 ±0.05

Figure Legends

1. Zinc Finger Nuclease (ZFN) targeted assay for iHR demonstrates increased iHR in FLT3-ITD mutated cells. A-B, Maps of targeting vectors. A, PZDonor-AAVS1 Puromycin, B, PZDonor. HA – homology arm. C, DR1 insert possesses a 5' GFP sequence interrupted by an 18bp recognition sequence for *I-SCE*I. DR1 insert was cloned into the MCS of pZDonor-AAVS puromycin vector to create PZD-DR1-Puro. D, DR2-BLAST insert possesses a truncated 3' GFP sequence. The construct was cloned into the MCS of pZDonor to create PZD-DR2-Blast. E, To confirm PZD-DR1-Puro and PZD-DR2-Blast integration at separate AAVS1 loci, vector and ZFN transfected cells were aliquoted into 24 well plates and individually checked for integration using PCR with vector and locus specific primers. Correct integration would produce a PCR product for PZD-DR1-Puro of 2300bp (upper panel) and PZD-DR2-Blast of 2100bp (bottom panel). F,G Co-FISH using a fluorescein labelled PZD-DR1-Puro (green) and Texas Red labelled PZD-DR2-Blast. Probes were sequentially hybridised to (F) DAPI stained cells and (G) metaphase chromosomes. Original magnification X 100. H, FACS analysis of MOLM-13PZD, MOLM-13PZD pre-treated with 25nM AC220 and FLT3-ITD transfected HEK293PZD. To determine GFP recombination events, cells were analysed using green (FL1) and orange (FL2) filters. Percentage of recombined GFP positive cells (recombination events) were made relative to transfection of a targeted GFP expressing plasmid. Top panel shows GFP+ve MOLM-13PZD cells, middle panel shows GFP+ve MOLM-13PZD cells with prior AC220 addition, bottom panel shows FLT3-ITD transfected HEK293PZD GFP+ve cells. I-J, Restriction digest of PZD-DR1-Puro PCR products indicates method of repair. Only GFP positive cells would give *BCG*1 digestible PCR products. J, PCR products derived from GFP recombined events from 2 separate experiments were digested with I-*SCE*I or *BCG*1. PCR products derived from MOLM-13PZD GFP recombination events were digestible with *BCG*1 and not I-*SCE*I.

2. Mutant FLT3-ITD increases HR activity and events in primary NK-AML. A, FACS analysis of PZD-DR1/DR2-Puro targeted cells in primary FLT3-ITD NK-AML (NK4). GFP positive cells (recombination events) were analysed using green (FL1) and orange (FL2) filters. Recombination events were made relative to transfection of a targeted GFP expressing plasmid. B,C Expression of DNA damage and growth signalling proteins were analysed by western blotting. Immunoblots were prepared from B) whole cell extracts of primary NK-AML (NK4) pre-treated with AC220 for 4h prior to harvest and C) whole cell extracts of transfected U937 with WT-FLT3, FLT-ITD or empty vector. Tubulin acted as a loading control. D-F, HR repair analysed by immunohistochemistry for phospho-γH2AX and RAD51 foci. D, Composite figure of foci formation in FLT3-ITD mutated primary NK-AML cells (NK-10). Original magnification X 100. E, Frequency of cells displaying greater than 5 foci per cell in primary AML. F, Graph of relative ROS % in primary NK-AML. Error bars represent the means ± S.E.M. of three separate experiments. G,H SCE analysis of primary NK-AML. G, Metaphase spreads of SCE from primary NK-AML cells (NK4). H, Graph of SCE. Error bars represent the means ± S.E.M. of three separate experiments. At least 50 cells in metaphase were counted per test. Error bars represent the means ± S.E.M. of three separate experiments. Original magnification X 100.

3. Mutant JAK2V617F confers iHR associated with increased HR activity and ROS. A-B, RAD51 expression by Q-PCR and western blotting in HEL (JAK2V617F), U937 (WT JAK2), HEK293 (WT JAK2) + Empty vector (EV), HEK293 + WT JAK2, HEK293 + JAK2V617F. A, cDNA relative target abundance (fold change) was normalized against the house-keeping genes GAPDH, Tubulin and B2M and then normalized to JAK2 expression. B, Immunoblots were prepared from whole cell extracts of HEL cells and WT-JAK2, JAK2V617F or empty vector transfected HEK293. Tubulin acted as a loading control. C,D iHR and HR activity in JAK2V617F mutated cells. C, iHR events in JAK2V617F transfected HEK293PZD. Cells were pre-treated with NAC where indicated for 5 days prior to analysis. Left panel shows GFP+ve cells without NAC, right panels show GFP+ve cells with NAC pre-treatment. D, HR

events in PZD-DR1/DR2-Puro targeted cells HEL cells (upper panel) and JAK2V617F transfected HEK293 (lower panel). To determine DR-GFP recombination events, cells were analysed using green (FL1) and orange (FL2) filters. Percentage of recombined GFP positive cells (recombination events, inset %) was made relative to transfection of a targeted GFP expressing plasmid. E, Frequency of cells displaying greater than 5 foci per cell and D, number of foci per cell for RAD51 foci (%) (Red bars) in primary cells and transfected cell lines. 200 nuclei were counted per experiment. Error bars represent the means ± S.E.M. of three separate experiments. F, Graph of relative ROS % in WT JAK2 or JAK2V617F cells. Error bars represent the means ± S.E.M. of three separate experiments.

4. G_0 arrested FLT3-ITD mutants demonstrate RAD51 expression and significant HR activity. A,B Composite representation of RAD51 foci in A, G_0 arrested primary FLT3-ITD NK-AML and B, G_0 arrested primary WT FLT3 NK-AML by immunocytochemistry. Original magnification X 40. C,D Identification of RAD51 expression in C, G_0 arrested primary NK-FLT3-ITD AML and D, G_0 arrested primary WT FLT3 NK-AML by western blotting. Immunoblots were prepared from whole cell extracts of primary NK-AML and probed with anti-RAD51. Tubulin acted as a loading control. E, Cell cycle kinetics of G_0 arrested MOLM-13 and U937. Arrested cells determined as G_0 phase (histogram peak and dot plot bottom left quadrant cells (insert)) were collected by FACS sorting. F. G_0 and S/G_2 FACS sorted cells were analysed for RAD51 expression by western blotting in MOLM-13 and U937. Tubulin acted as a loading control. G, FACS analysis of PZD-DR1/DR2-Puro transfected G_0 arrested MOLM-13 with and without prior treatment with AC220 or NAC. To determine HR events, cells were analysed using green (FL1) and orange (FL2) filters. Percentage of recombined GFP positive cells (recombination events) was made relative to transfection of a targeted GFP expressing plasmid.

5. Spontaneous thymidine kinase (TK) mutation rate and frequencies of TK6 cells transfected with FLT3-ITD and WT FLT3 expressing vectors or controls. A, Mutation rate and frequencies in transfected TK6 cells treated with and without AC220 or NAC. Error bars

represent the means ± S.E.M. of three separate experiments. B, Mutation spectrum at the TK locus. Fragment fluorescent PCR was used to determine the presence of LOH. Mutants with double peaks (left panel) for each exon were considered wild type at the TK locus. Mutants with single peaks have LOH. Comparison of peak areas relative to the peak area for β-globin can be used to distinguish copy neutral LOH (CN-LOH) and hemizygous LOH. CN-LOH (right panel) will have double peak area compared to hemizygous LOH. C, Graph of CN-LOH TK mutants from transfected TK6 treated with and without AC220 or NAC. Error bars represent the means ± S.E.M. of three separate experiments. D, Determination of CN-LOH regions at the TK locus. Fluorescent fragment PCR of heterologous microsatellites at the TK locus were analysed to determine the extent of LOH in FLT3-ITD TK mutant clones. Approximate position of each microsatellite is stated. Human TK locus maps to 17q25. Length of bars indicates size of CN-LOH at the TK locus. E, Proposed model of the collaborative activities of ROS and HR to create CN-LOH in mutated FLT3 myeloid malignancy.