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Loss of MLH1 expression promotes the acquisition of oncogenic JAK1 and JAK3 mutations that cooperatively increase resistance to JAK inhibitors

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vaincre la leucémie et
les maladies du sang

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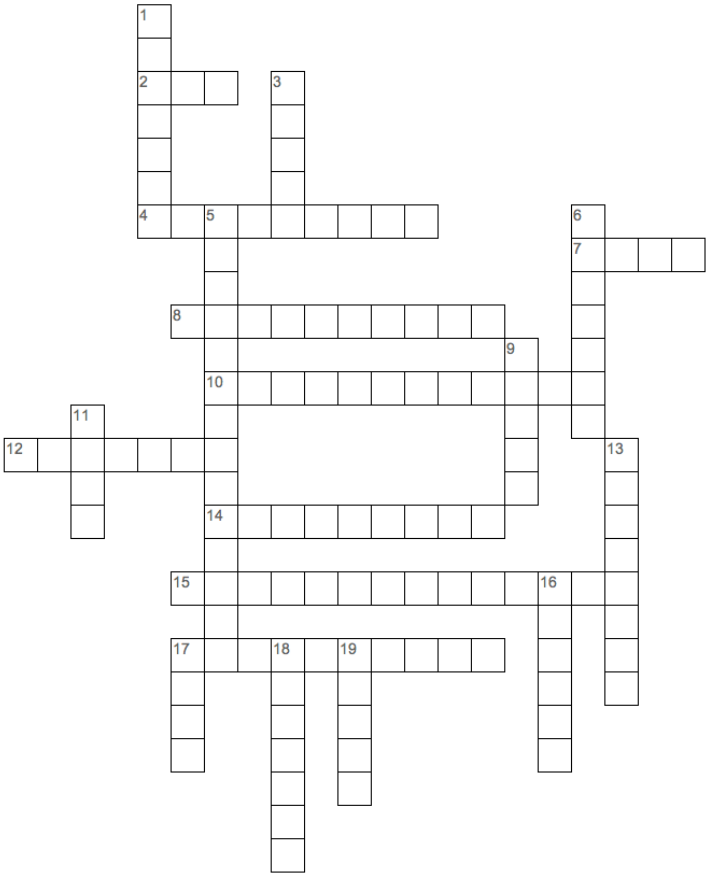
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SUMMARY

Malignant transformation evolves as the outcome of 2 connected processes operating within a population of competing cells: on the one hand, the continuous accumulation of random mutations by individual cells at a rate dictated by the degree of genetic instability and, on the other hand, the simultaneous natural selection acting on the resultant phenotypes. Successive rounds of environmental pressures lead to the emergence of cells that acquired diverse advantageous hallmarks such as growth factor-independent proliferation, mostly driven by constitutively activated signaling cascades to which neoplastic cells develop an addiction that can be therapeutically exploited. For instance, the JAK-STAT pathway is frequently hyperactivated in T-ALL and the use of JAK inhibitors in lymphoid malignancies is currently under pre-clinical investigations. In the present thesis, we took advantage of the TS1 cell line recapitulating in vitro JAK-STAT pathway-driven T-cell transformation in order to unravel the underlying genetic events and evaluate the efficacy of JAK inhibitors.

In our first study, we found that growth factor-independent TS1 clones acquired an activating point mutation in JAK1 and/or JAK3, which function as partners in the signal transduction downstream of γc-sharing cytokine receptors. Furthermore, TS1 cells transformed by an activated mutant of JAK1 became resistant to JAK inhibitors by acquiring a secondary mutation in JAK3 and *vice versa*. We demonstrated that the presence of two inhibitor-sensitive JAK1 and JAK3 mutants cooperatively activate STAT3 and STAT5 factors thereby increasing the resistance to JAK inhibitors.

In our second study, we focused on the molecular mechanism underlying the high rate of spontaneous mutagenesis in TS1 cells. We demonstrated that loss of MLH1 expression, a key component of the mismatch repair system, promotes the occurrence of oncogenic point mutations in JAK kinases driving transformation and acquired resistance to inhibitors. We confirmed the clinical relevance of our

findings by showing that chronic myeloid leukemia cells from patients that relapsed upon ABL-targeted therapy have a lower expression of MLH1 messenger RNA than leukemic cells from patients at diagnosis. Moreover we identified a particular mutational signature present in our TS1 cell model and corresponding to the one described in primitive CD32+ cells from chronic myeloid leukaemia patients that might attest MLH1-deficiency. Taken together, our findings suggest that MLH1 status should be considered in order to predict response to targeted therapies.

ABBREVIATIONS

6-TG	6-Thioguanine
AMKL	Acute Megakaryoblastic Leukemia
AML	Acute Myeloid Leukemia
B-ALL	B-cell Acute Lymphoblastic Leukemia
BAT	Best Available Therapy
BER	Base Excision Repair
bMMRD	bi-allelic Mismatch Repair Deficiency
BTK	Bruton Tyrosine Kinase
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CNL	Chronic Neutrophilic Leukemia
DS-ALL	Down Syndrome-associated ALL
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
EPO	Erythropoietin
ET	Essential Thrombocythemia
ETP-ALL	Early T-cell Precursor ALL
FDA	Food and Drug Administration
FERM	band 4.1, Ezrin, Radixin, Moesin
FLT3	Fms-Like receptor Tyrosine kinase 3
G-CSF	Granulocyte Colony-Stimulating Factor
GF	Growth Factor
GIST	Gastro-Intestinal Stromal Tumors
GM-CSF	Granulocyte/Monocyte Colony-Stimulating Factor
HL	Hodgkin Lymphoma
HNPCC	Hereditary Non-Polyposis Colon Cancer
HPRT	Hypoxanthine-guanine Phosphoribosyl Transferase
HSC	Hematopoietic Stem Cell
IDL	Insertion-Deletion Loop
IFN	Interferon
IL	Interleukin
JAK	Janus Kinase
JH	JAK Homology region
JMML	Juvenile Myelomonocytic Leukemia

LGL	Large Granular Lymphocyte Leukemia
MAPK	Mitogen-Activated Protein Kinase
MDS	Myelodysplasic Syndrome
MLH1	MutL Homolog-1
MMR	Mismatch Repair
MPN	Myeloproliferative Neoplasm
MSC	Mesenchymal Stromal Cells
MSH	MutS-Homolog
MSI	Microsatellites Instability
MTOR	Mammalian Target of Rapamycin
NER	Nucleotide Excision Repair
NGS	Next-Generation Sequencing
NSCLC	Non-Small Cells Lung Cancer
PCNA	Proliferating Cell Nuclear Antigen
PDGFR	Platelet-Derived Growth Factor Receptor
PI3K	Phosphatidylinositol-3'-Kinase
PMBCL	Primary Mediatsinal B-Cell Lymphoma
PMF	Primary Myelofibrosis
PMS	Post-Meiotic Segregation
PTP	Protein Tyrosine Phosphatase
PV	Polycythemia Vera
R	Receptor
RFC	Replication Factor C
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
SH2	Sarc Homology-2
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SOCS	Supressor Of Cytokine Signaling
STAT	Signal Tranducer and Activator of Transcription
T-PLL	T-cell Prolymphocytic Leukemia
TKI	Tyrosine Kinase Inhibitor
TMD	Transmembrane Domain
TPO	Thrombopoietin
TSLP	Thymic Stromal-derived Lymphopoietin
TYK2	Tyrosine Kinase 2

INTRODUCTION

INTRODUCTION

1. JAK kinase targeting in hematological malignancies: A sinuous pathway from identification of genetic alterations towards clinical indications

1.1 Introduction

Mature blood cells have a limited lifespan and are continuously renewed through a multi-step process called hematopoiesis, initiated in the bone marrow by the proliferation and differentiation of a small population of pluripotent hematopoietic stem cells (HSCs) (Figure 1). Undergoing asymmetric divisions, HSCs have the ability to replenish their pool by self-renewal and to differentiate into lineage-committed progenitors with increasingly restricted potential that will ultimately give rise to all specialized blood cells[1]. A network of hematopoietic cytokines tightly dictates fate (proliferation, differentiation or apoptosis) of the various progenitors allowing the maintenance of steady state blood cells level in the periphery or the amplification of specific cell types in response to a particular stimulus to meet physiological needs. Abnormalities in the hematopoietic program disrupt homeostasis and drive the accumulation of intermediate progenitors and/or mature cells in the bone marrow, blood and/or peripheral lymphoid organs resulting in a variety of malignancies[2].

Overview of the JAK-STAT pathway

Hematopoietic cytokines bind at the surface of target cells to their cognate receptors composed of at least two single membrane-spanning chains. Except several tyrosine kinase receptors like c-Kit, Fms-like tyrosine kinase 3 (FLT3) or the receptor of macrophage colony-stimulating factor (M-CSF), the intracellular part of hematopoietic receptor chains lacks intrinsic enzymatic activity. However, these receptor chains constitutively and specifically associate with a member of the Janus kinase family (JAK1, JAK2, JAK3 or TYK2) in order to form functional

complexes able to transduce ligand-induced signals. Following cytokine engagement, receptor chains reorientate or oligomerize leading to juxtaposition, and hence transactivation of the 2 associated JAKs. Once activated, JAKs phosphorylate tyrosine residues on themselves and in the cytoplasmic part of the receptor creating docking sites for downstream Src homology-2 (SH2) domain-containing adaptors and effectors proteins. Depending on the amino acids surrounding the phosphotyrosine, any one or more of the 7 signal transducer and activator of transcription factors (STAT-1, -2, -3, -4, -5a, -5b and -6) can be recruited and phosphorylated at the receptor, homo- or heterodimerize and translocate into the nucleus to regulate transcription of target genes[3].

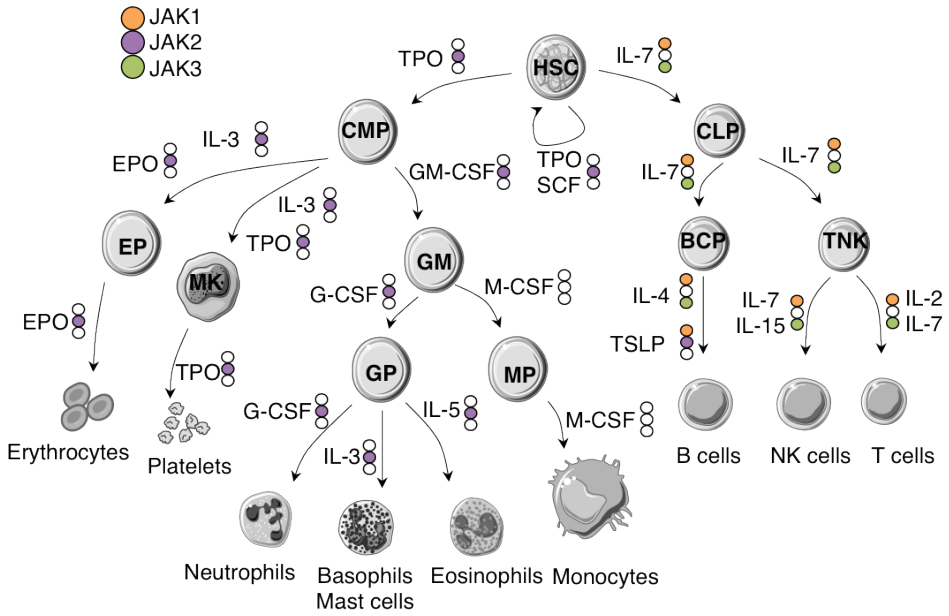


Figure 1. Hematopoiesis. Hematopoiesis originates from a hematopoietic stem cell, which can undergo either self-renewal or hierarchical differentiation into lineage-committed progenitors with decreasing potential that ultimately will give rise to all mature blood cells. Cytokines and their receptor-associated JAK necessary for the progenitors to pass through the different maturation steps are indicated. (HSC) Hematopoietic stem cell, (CMP) Common myeloid progenitors, (CLP) Common lymphoid progenitors, (GM) Granulocyte macrophage progenitors, (BCP) B cell progenitor, (TNK) T and Natural killer cells progenitors, (EP) Erythroid progenitors, (Mk) Megakaryocyte, (GP) Granulocyte progenitors, (MP) Macrophage progenitors, (TPO) Thrombopoietin, (SCF) Stem Cell Factor, (IL) Interleukin, (GM-CSF) Granulocyte/Monocyte Colony-Stimulating Factor, (G-CSF) Granulocyte-CSF, (M-CSF) Monocyte-CSF, (TSLP) Thymic Stromal-Derived Lymphopoietin.

Introduction

The JAK-STAT pathway constitutes a signal transduction system where a large spectrum of extracellular cytokines and nearly as many cognate transmembrane receptors converge towards an intracellular code employing 4 JAK kinases and 7 STAT factors[4]. Signal specificity downstream of cytokine receptors is achieved by the nature of the STAT dimers formed at the receptor, the kinetics and intensity of STAT activation as well as the triggering of additional signaling pathways such as the mitogen-activated protein kinases (MAPK) and the phosphatidylinositol-3'-kinase (PI3K). Transient JAK-STAT pathway activation is achieved by several mechanisms of negative regulation that operate at each step of signal transduction such as ubiquitin-mediated receptor internalization, dephosphorylation of tyrosines in the JAK activation loop by constitutive phosphatases and induction of Suppressor of Cytokine Signaling (SOCS) proteins[5].

In 2005, several groups reported a unique acquired somatic activating mutation of JAK2 (V617F) in 95% of Polycythemia Vera patients (PV) and in about half of Essential Thombocythemia (ET) and Primary Myelofibrosis (PMF) patients[6-9]. The discovery of JAK2 V617F gave an impetus for the screening of JAK mutations in other hematological neoplasms. Thanks to improvement of sequencing techniques and the conduction of massive sequencing projects, the catalogue of genetic alterations in hematological malignancies is constantly updated as still more and more new mutational targets are discovered. This review will depict an overview of the different molecular mechanisms underlying constitutive activation of the JAK-STAT pathway and their respective frequencies among hematological neoplasms. The use of JAK inhibitors in the clinic and in ongoing trials, resistance phenomena, as well as future challenges will be discussed. This chapter will begin by documenting insights into the structural organization of JAK kinases, their mode of activation as well as the role of the different family members in hematopoiesis.

Janus kinases

JAK1, JAK2 and TYK2 tyrosine kinases are ubiquitously expressed and non-covalently bound to a distinct, mostly non-overlapping repertory of receptor chains. By contrast, JAK3 expression is restricted to the hematopoietic lineage and it associates exclusively with the common gamma-chain (γc). Based on their primary sequences, JAK kinases were initially divided into 7 highly conserved JAK Homology regions (JH1-7, starting from the C-terminal end) but were thereafter organized in 4 functional domains[10].

The N-terminal moiety of JAK kinases constitutes the receptor-binding module with a FERM (band 4.1, Ezrin, Radixin, Moesin) domain (JH4-7) and an atypical SH2 domain (JH3)[11]. It has been experimentally established that both domains do not exist as individual functional entities but structurally cooperate for non-covalent anchoring to two membrane-proximal regions of defined receptors[12,13]. This was corroborated by a recent crystal structure study showing that the N-terminal part of TYK2 forms a contiguous, Y-shaped receptor-binding module that interacts with the interferon alpha receptor 1 (IFNAR1) via a composite interface formed by the FERM and SH2 domains[14]. The selectivity of the JAK member engaged by a particular receptor chain does not exclusively depend on intrinsic sequences within its cytoplasmic domain but also on the stoichiometry of the receptor. For example, forced homodimers of chimeric receptors encompassing the intracellular part of IL-2Rβ fused to the extracellular part of EPO-R activated JAK2 despite the fact that the naturally activated kinase was JAK1[15]. Of note, JAK-receptor association is needed to ensure proper trafficking and localization of the complex to the plasma membrane[16-18].

The C-terminal moiety of JAKs contains the catalytic kinase domain (JH1), whose active conformation is stabilized upon phosphorylation of tandem tyrosine residues in its activation loop. Inappropriate kinase activity of JH1 in the absence of stimulus is prevented by a directly adjacent pseudo-kinase domain (JH2)[19]. The precise molecular mechanism by which JH2 regulates JAK activity remains incompletely understood but insights have been provided by several recent works[20]. The

pseudokinase domain has long been considered as catalytically inactive because it lacks critical conserved residues needed for phosphate coordination and transfer such as the nearly invariant aspartic acid in the catalytic site and the third glycine of the GxGxϕG motif in the ATP-binding loop. However, JAK2 JH2 crystal structure revealed that the pseudo-kinase domain adopts a classical bilobal kinase-fold and binds ATP in a noncanonical mode[21]. The precise function of the nucleotide binding of JH2 is still debated. JH2 has been demonstrated to phosphorylate two negative regulatory sites of JAK2, namely Ser523 in the SH2-JH2 linker and Tyr570 in JH2 itself[22]. Whether this weak kinase activity is relevant for autoinhibition is still a matter of controversy since those residues are not conserved among other family members and disruption of JH2 nucleotide binding does not lead to constitutive JAK2 activation. Rather, it has been proposed that JH2 constitutively binds ATP without hydrolysis, making ATP an essential structural stabilizer of JH2[23].

Study of recombinant JH1 and JH1-JH2 constructs from JAK2 showed that the presence of JH2 drastically decreases JH1 kinase activity by increasing the Km for ATP possibly via an interdomain interaction that changes JH1 conformation[24]. Owing to the recently solved crystal structure of JH1-JH2 from TYK2, this intramolecular inhibitory interface has been localized near the JH1 catalytic site and is predominantly mediated by the N-lobes of each domain[25]. The JH1-JH2 crystal structure of TYK2 supports a mode of inhibition *in cis* with the pseudokinase domain inhibiting the directly adjacent kinase domain within the same protein. Nevertheless, it is unclear whether this intramolecular JH1-JH2 interplay is operative in a physiological receptor context. Because cytokine receptor complexes crucially comprise two or more subunits each associated with a JAK monomer, one cannot exclude an *in trans* mechanism of inhibition where the pseudokinase domain of one JAK molecule locks the kinase domain of the opposing JAK. Recently, Brooks et al. used fluorescence resonance energy transfer (FRET) to monitor the movements of JAK2 JH1 and JH2 domains during stimulation of the homodimeric growth hormone (GH) receptor. Activation resulted in an increase in distance between the two JH2 while bringing the two JH1 in closer vicinity,

consistent with the *in trans* inhibition model[26]. Supporting evidences for both *in cis* and *in trans* inhibition models could be conciliated by the hypothesis that distinct mechanisms operate between different JAK family members or that the type of associated receptors governs the mode of regulation[27].

Role of JAK kinases in hematopoiesis

As outlined in figure 1, JAK2 plays a role in function and maintenance of HSCs[28] by participating directly to signal transduction of thrombopoitein (TPO)[29] and by contributing to signaling downstream of the stem cell factor (SCF)[30]. JAK2 is also crucial at various stages of myelopoiesis through binding to receptors of erythropoietin (EPO), TPO, granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5. The EPO receptor exists as a preformed homodimer[31], while those for TPO and G-CSF are believed to homodimerize upon ligand binding. Receptors for GM-CSF, IL-3 and IL-5 consist of heterodimers of a cytokine-specific α chain and the common β chain that assemble as ligand-induced tetramers. In all cases, signal transduction will result from transactivation of a pair of juxtaposed JAK2 molecules.
By contrast, JAK1 cooperates with JAK3 for lymphopoiesis by binding to heterodimeric receptors of the common γc family, IL-2R, IL-4R, IL-7R and IL-15R with JAK1 anchored to the specific chain and JAK3 to the common γc. Both kinases play obligatory and non-redundant functions as cells lacking either JAK1 or JAK3 are unable to respond to γc cytokines[32,33]. However, the catalytic activity of each partner is not of equal importance since kinase-dead form of JAK3 but not JAK1 still allows signal transduction via these receptors[34]. Therefore, in the context of γc-containing receptors, JAK1 functions as the primary signaling effector whereas JAK3 serves as scaffold and partner of JAK1. JAK2 in concert with JAK1 could possibly be implicated in late development of immature B-lymphocytes through signaling downstream of the thymic stromal-derived lymphopoietin (TSLP)[35]. In respect to their implication in the maturation and amplification of the different myeloid and lymphoid lineages (Figure 2), genetic alterations leading to

oncogenic activation of particular hematopoietic cytokine receptor complexes give rise to neoplasms of corresponding cell types.

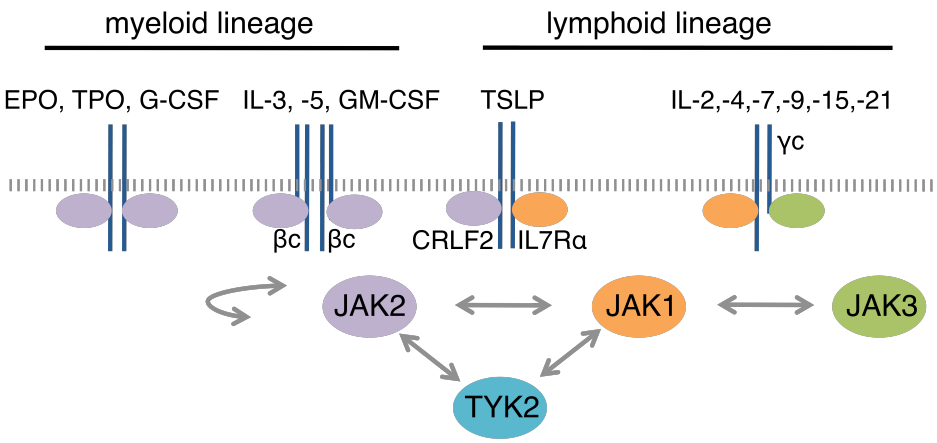
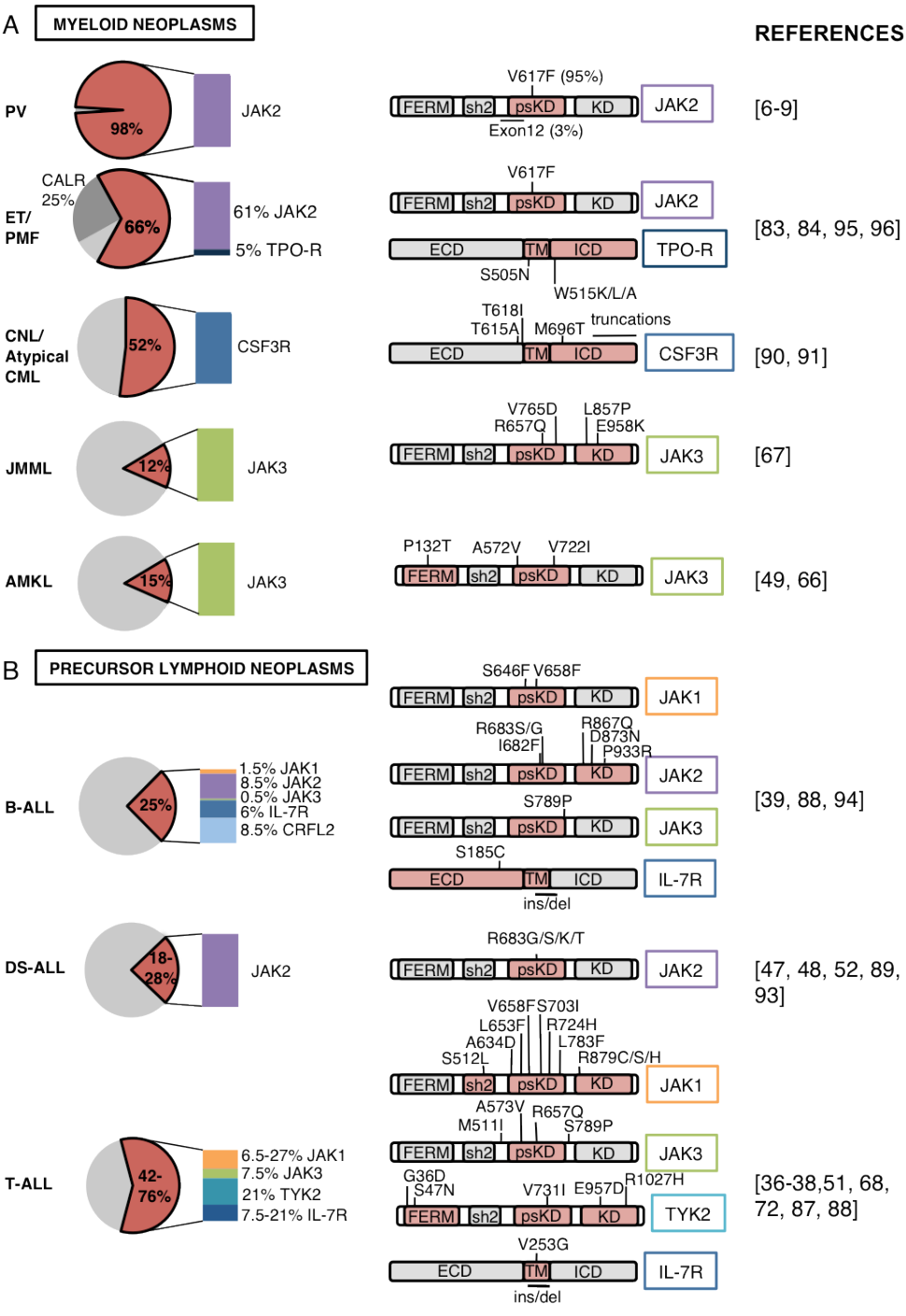


Figure 2. Hematopoietic cytokine-receptor complexes. Hetero- or homo-oligomerized receptor chains specifically engage a member of the JAK kinases in order to form signaling-competent hematopoietic receptor complexes. Myelopoiesis is driven by cytokines whose receptors signal through the transactivation of a pair of juxtaposed JAK2 molecules. Lymphopoiesis is mostly driven by interleukins that bind to the shared γc-receptor family, which signals through the cooperative action of JAK1 and JAK3. JAK2 and JAK1 participate to the signaling of TSLP, a cytokine involved in late development of B-lymphocytes. JAK1, JAK2 and TYK2 can work as partners whereas JAK3 works exclusively in concert with JAK1. JAK2 is the only family member that can function with itself as opposing JAK. (EPO) Erythropoietin, (TPO) Thrombopoietin, (G-CSF) Granulocyte-colony stimulating factor, (IL) Interleukin, (GM-CSF) Granulocyte/monocyte-CSF, (TSLP) Thymic stromal-derived lymphopoietin.

1.2 Activating alterations of JAK kinases

Hematological malignancies have been associated with activating alterations in the four members of the JAK family at different frequencies, the majority of them being point mutations. Contrasting with the dominance of the JAK2 V617F mutation in myeloproliferative neoplasms (MPN), the mutations described later in other hematological malignancies in JAK1, JAK2, JAK3 and more recently in TYK2 are much more heterogeneous both regarding the mutated residues and the frequency

of mutation in a given disease. The JAK2 locus can also be implicated in translocations resulting in the overexpression of wild-type JAK2 or the generation of constitutively active fusion proteins. Figure 3 gives a general outlook of the different myeloid and lymphoid neoplasms for which the overall frequency of analyzed cases with genetic alterations affecting any component of the cytokine receptor-JAK-STAT axis reached at least 10%.


A
MYELOID NEOPLASMS
REFERENCES
PV
98%
JAK2
V617F (95%)
FERM sh2 psKD KD
JAK2
Exon12 (3%)
[6-9]
CALR 25%
ET/ PMF
66%
61% JAK2
5% TPO-R
V617F
JAK2
ECD TM ICD
TPO-R
S505N
W515K/L/A
[83, 84, 95, 96]
CNL/ Atypical CML
52%
CSF3R
T618I
T615A
M696T
truncations
CSF3R
[90, 91]
JMML
12%
JAK3
V765D
R657Q
L857P
E958K
[67]
AMKL
15%
JAK3
P132T
A572V
V722I
[49, 66]
B
PRECURSOR LYMPHOID NEOPLASMS
B-ALL
25%
1.5% JAK1
8.5% JAK2
0.5% JAK3
6% IL-7R
8.5% CRFL2
S646F V658F
JAK1
R683S/G
I682F
R867Q
D873N
P933R
S789P
S185C
ins/del
IL-7R
[39, 88, 94]
DS-ALL
18-28%
JAK2
R683G/S/K/T
[47, 48, 52, 89, 93]
T-ALL
42-76%
6.5-27% JAK1
7.5% JAK3
21% TYK2
7.5-21% IL-7R
V658F S703I
L653F
R724H
A634D
L783F
S512L
R879C/S/H
A573V
R657Q
M511I
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[36-38,51, 68, 72, 87, 88]

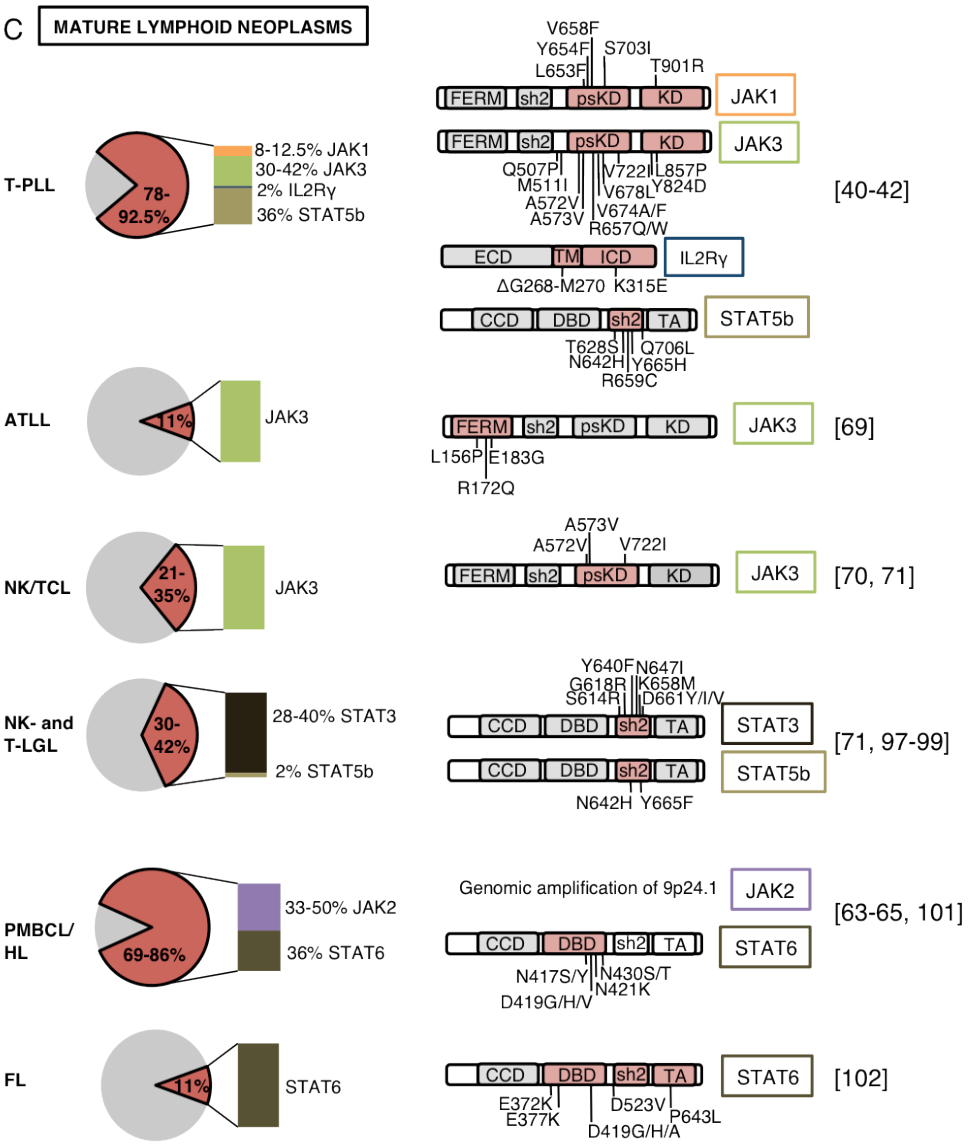


Figure 3. Frequencies of genetic alterations affecting the receptor-JAK-STAT axis among hematological malignancies. Diagrams give an overview of the maximal overall frequency of cases with genetic alterations of the receptor-JAK-STAT axis in (A) myeloid lineage, (B) lymphoid progenitors and (C) lymphoid mature cells disorders. The overall frequency of JAK-STAT pathway mutations (i.e. the sum of the individual mutation frequencies for each component) is indicated in red while the absolute contributions of each are detailed using a specific colour code (yellow for JAK1, violet for JAK2, green for JAK3, turquoise for TYK2, blue for cytokine receptors, black for STAT3, beige for STAT5b and brown for STAT6). Schematic protein representations are used to localize the identified point

mutations with the affected functional domains indicated in red. (MPN) Myeloproliferative neoplasms, (PV) Polycythemia vera, (ET) Essential thrombocythemia, (MF) Myelofibrosis, (CNL) Chronic neutrophylic leukemia, (CML) chronic myeloid leukemia, (JMML) juvenile myelomonocytic leukemia, (AMKL) Acute megakaryoblastic leukemia, (ALL) Acute lymphoblastic leukemia, (DS) Down-syndrome, (T-PLL) T-prolymphocytic leukemia, (ATLL) adult T-cell lymphoma, (NKTCL) NK/T-cell lymphoma, (NK- and T-LGL) NK- and T-large granular lymphocytic leukemia, (PMBL) Primary mediastinal B-cell lymphoma, (HL) Hodgkin lymphoma, (FL) Follicular lymphoma.

Frequencies of alterations in JAK kinase genes among hematological malignancies

In accordance with its role in lymphopoiesis, activating mutations of JAK1 were described in lymphoid neoplasms with the highest frequency in T-cell acute lymphoblastic leukemia (ALL) (6.5-27%)[36-38] and lower frequencies in B-ALL (1.5%)[39] and T-cell prolymphocytic leukemia (T-PLL) (8-12.5%)[40-42]. JAK1 mutants were also reported in very rare cases of acute myeloid leukemia (AML)[43].

As already mentioned, the JAK2 V617F mutation is highly prevalent in classical MPNs so that its screening has become a standard diagnostic procedure in the clinic. The same JAK2 V617F mutation was described later in a small proportion of patients with atypical MPNs[44], and in some cases of myelodysplastic syndromes (MDS), particularly in patients suffering from refractory anemia with ringed sideroblasts and thrombocythemia[45]. In addition, mutations in the exon 12 of JAK2 were described in JAK2 V617F-negative PV patients who typically harbored isolated erythrocytosis[46].

JAK2 mutations were also found in lymphoid malignancies such as high-risk B-ALL (8.5%)[39] and B-ALL associated with Down's syndrome (18-28%)[47-53]. Noticeably, the vast majority of lymphoid lineage JAK2 mutations affects the R683 residue in JH2. Interestingly, JAK2 R683 variants are frequently associated with the overexpression of CRLF2, suggesting that this cytokine receptor chain is involved in the functional signaling platform of these mutants[54]. CRLF2 is a JAK2-binding chain that forms together with IL-7Rα and JAK1 the functional receptor complex for thymic stromal-derived lymphopoietin (TSLP). The rationale for the specific association of $JAK2^{V617F}$ with MPN and $JAK2^{R683}$ with ALL is speculative. The $JAK2^{V617F}$ mutant might be more efficient in transactivating another JAK2 in homodimeric cytokine receptor complexes such as EPO-R or TPO-R, whereas

conversely the JAK2^{R683} variants might preferentially activate a JAK1 molecule in the heterodimeric TSLP receptor complex. Alternatively, the two mutants might differ in their affinity for a particular receptor chain, substrate specificity or intensity of signal transduction. Finally, hypermutability of the V617 and R683 hot spots might be influenced by cell type-dependent factors such as secondary DNA structures, the chromatin context of adjacent regions or specific replicative polymerases.

Rare chromosomal rearrangements juxtaposing the kinase domain-coding portion of the JAK2 gene to genomic regions coding for the oligomerization domain of either TEL, BCR, PCM1, Pax5 or ETV6 were described essentially in ALL and several cases of atypical chronic myeloid leukemia (CML)[51,55-59]. The resultant fusion proteins oligomerize, facilitating transphosphorylation of the JAK2 activation loops, and constitutively trigger several downstream signal transduction pathways, such as STAT5[55], PI3K[60] or the MAP kinases[61] independently of the presence of anchoring receptors. Interestingly, although artificial chimeric TEL-JAK1, TEL-JAK3 and TEL-TYK2 proteins are able to sustain cytokine-independent growth of Ba/F3 cells[62], there is no patient-derived chromosomal translocation that fuses the kinase domain of JAK1, JAK3 or TYK2 to a dimerizer described so far. This is probably related to an intrinsic genetic instability of the JAK2 locus, which can otherwise also be subject to amplifications in 30-50% of Hodgkin lymphomas (HL) and primary mediastinal B-cell lymphomas (PMBCL)[63-65].

Gain-of-function mutations in JAK3 are scattered throughout the different functional domains and are found in diverse malignancies from both myeloid and lymphoid origins. JAK3 mutations were initially described in acute megakaryoblastic leukemia samples (AMKL) (15%)[66] and were subsequentially identified in juvenile myelomonocytic leukemias (JMML) (12%)[67]. Concerning the lymphoid lineage-malignancies, JAK3 mutations were reported in a significant proportion of T-ALL[68], especially in an aggressive subtype called early T-cell precursor-ALL (ETP-ALL)[38], as well as in diverse mature T-cell neoplasms [40,42,69-71].

More recently, TYK2 mutants were discovered in T-ALL (21%) and seem to

promote cell survival via activation of STAT1 downstream of IL-10R and upregulation of the anti-apoptotic BCL2 protein[72].

Characterization of JAK mutants

Functional characterization of the different JAK variants revealed that they display common features as well as mutant-specific particularities. As demonstrated for JAK1, JAK2 and JAK3, mutants require a functional FERM domain and the presence of anchoring homo- or heterodimeric cytokine receptors to promote signaling and cell transformation[73-75]. One notable exception is the JAK1 L910P mutant that exhibits residual activity upon inactivating mutation in its FERM-domain[75]. JAK1 mutants can trigger STAT activation via IL2-Rβ or IL-9Rα homodimers without need for JAK3 and γc[74]. By contrast, JAK3 mutants (with the exception of JAK3 L857Q) are strictly dependent on JAK1 kinase activity[76]. Intrinsic specificity among distinct mutants of the same JAK is illustrated by qualitative and quantitative differences in STAT activation, FERM-domain requirement, susceptibility to JAK inhibitors and, concerning the JAK1 mutants, sensitivity to type-I interferons[75-78].

The overwhelming majority of activating JAK mutations occur in the pseudokinase domain (JH2). The mechanism through which such mutations increase the activity of the kinase domain was almost exclusively studied for JAK2 V617F. Superimposing the crystal structures of the pseudokinase domains of JAK2 V617F and the analogous JAK1 V658F mutants with their wild-type counterparts highlighted a stiffening and extension of the αC helix mediated by the action of a phenylalanine triad[21,79]. The aromatic ring of the phenylalanine at position 617 in JAK2 and 658 in JAK1 fills in the site normally occupied by that of a highly conserved phenylalanine residue in the SH2-JH2 linker (F537 in JAK2 and F575 in JAK1) thereby inducing the rotation of a third phenylalanine located in the αC helix (F595 in JAK2 and F636 in JAK1). Replacement of either partner of this F-F-F triad by non-aromatic residues diminishes constitutive kinase activity[79,80]. Interestingly, JAK2 V617F is transferable to JAK1 and TYK2 but not JAK3 evoking a different

autoinhibition mechanism of JAK3 compared to the three others[81].

Besides JAK2 V617F, most of the cancer-associated mutations in the pseudokinase domain of JAK1, JAK2 and JAK3 are clustered in the N-lobe and the SH2-JH2 linker fragment. Mapping of the analogous affected residues on the crystal structure of TYK2 revealed that they localize in or near the JH1-JH2 interface and probably relieve the inhibitory influence[25]. On the other side of the interface, all known kinase N-lobe mutations participate directly to the JH1-JH2 interaction.

1.3 Activating alterations in cytokine receptors

The first mechanism of constitutive receptor activation involves point mutations or small insertions/deletions of residues located in and around the transmembrane domain (TMD) that result in unliganded chains reorientation or dimerization. Several examples are listed hereunder. TPO-R possesses an intracytoplasmic juxtamembrane amphipathic motif necessary for preventing its activation in the absence of ligand[82]. Mutations in this motif (W515K/L/A) were described in 5% of JAK2 V617F-negative MF and ET patients[83,84] and were shown to change the spatial conformation of the receptor enabling TPO-independent JAK2 transphosphorylation[85]. Additionally, mutations in the TMD of TPO-R (S505N and S505A) cause familial ET[86]. Small in-frame insertions/deletions in the TMD of the IL-7 receptor α-chain (IL-7Rα) were found in about 10 % of T- and B-ALL[87,88]. These sequence modifications often introduce an unpaired cysteine in the TMD and promote formation of intermolecular disulfide bonds between IL-7Rα mutant subunits, thereby driving constitutive signaling via JAK1 and independently of IL-7, γc or JAK3[87]. Point mutations inserting a cysteine in the extracellular domain of IL-7Rα (S185C) and CRLF2 (F232C) were identified in few B-ALL cases and could act via the same activation mechanism[89]. Membrane-proximal mutations of CSF3R (T615A and T618I), the receptor for G-CSF, are highly prevalent in atypical CML and in chronic neutrophilic leukemia (CNL), and induce ligand-independent activation of the receptor by a mechanism that remains to be elucidated[90,91]. More

recently, a whole-exome sequencing study provided the first report of 2 concomitant sequence modifications in γc (a small deletion in the TMD and the K315E mutation in the intracellular part) detected in a sample of T-PLL[41]. The significance of this finding is not yet clear as only one patient in a series of 50 analyzed samples harbored these γc mutations.

A second mechanism of receptor activation has been described in patients with CNL or atypical CML and involves truncations of the cytoplasmic tail of CSF3R, leading to escape of negative regulation[90]. Those truncated proteins are the result of frameshift or nonsense mutations and increase the proliferative signal by impairing receptor internalization and altering the interaction with negative regulators such as SOCS3. Interestingly, proliferation of cells transformed with these mutants was relatively insensitive to JAK2 inhibitors, but could be efficiently blocked by SRC inhibitors, such as dasatinib, indicating that SRC signaling could be preferentially activated by these truncated proteins. CSF3R truncating mutations were very recently identified in 2% of pediatric AML[92].

The last reported cytokine receptor alteration is the overexpression of CRLF2 that has been already mentioned in this review. CRLF2 is aberrantly expressed in 50 % of patients with DS-ALL and in 10 % of non-DS ALL[54,89,93,94], essentially owing to chromosomal rearrangements juxtaposing the CRLF2 locus to the immunoglobulin heavy chain enhancer or P2RY8 promoter. In B-ALL, CRLF2 overexpression in frequently associated with JAK2 R683 variants or IL-7Rα mutations, suggesting that concomitant genetic events affecting components of the TSLP-receptor complex are required for its oncogenic potency.

Taken together, gain-of-function alterations of cytokine receptors are diverse and encompass mutations in TM region that induce ligand-independent receptor dimerization/activation, cytoplasmic tail truncations that abolish negative regulation and chromosomal translocations that enhance protein expression. At present, such genetic modifications are well established for TPO-R, CRLF2, IL-7Rα and CSF3R but it is not excluded that whole-exome sequencing studies will further highlight alterations in additional cytokine receptor chains as suggested by the recent finding

of very rare mutations in γc.

Calreticulin mutations in JAK2 and TPO-R mutation-negative ET and MF

In December 2013, the molecular diagnostic gap that remained after the discovery of TPO-R mutations in JAK2-negative ET and MF patients has been partially filled in. Two independent groups reported that 25% of ET and MF patients conveyed small insertions/deletions in an unexpected gene coding for the calreticulin, a lectin chaperone involved in the folding quality control machinery of the endoplasmic reticulum (ER) and in calcium homeostasis[95,96]. All identified sequence modifications invariably provoked a +1 base shift to the same alternative open reading frame resulting in the generation of a novel C-terminus with impaired calcium-binding and loss of the ER-retention motif, although CALR WT and mutant forms did not show any difference in the pattern of subcellular localization[95]. Expression of the most frequent CALR mutant caused transformation to cytokine-independent cells with phosphorylation of STAT5 by means of a still unknown mechanism but the homogeneity within the frameshifted variants evokes a strong selective pressure towards newly acquired functionalities rather than loss-of-function alterations[96]. JAK2, TPO-R and CALR mutations were mutually exclusive genetic events in MPN with the remaining 10% triple negative being associated to the worse prognosis.

1.4 Activating mutations in STAT factors

For the moment, STAT3 and STAT5b mutations were only demonstrated in T- and NK-cell leukemias, while mutations of STAT6 have been associated with B-cell lymphomas. However, given their crucial function at all hematopoietic stages, it can be expected that further sequencing studies will emphasize the role of STAT family genes mutations in the pathogenesis of additional hematological malignancies from different origins.

STAT3 mutations

The occurrence of STAT3 mutations stressed the common molecular pathogenesis of chronic lymphoproliferative disorders of NK cell and T-cell large granular lymphocyte leukemia (NK- and T-LGL) as similar percentages (27-30%) of patients from both disease entities convey these somatic mutations[97,98]. Altogether, 8 different substitutions clustered in the exon 21 coding for the SH2 domain were identified and all affected residues mapped at the interface of STAT dimers. *In vitro* studies showed that Y640F and D661V mutants are constitutively phosphorylated and localize in the nucleus. Patients with STAT3 mutations presented more often with disease-related autoimmune phenomena like rheumatoid arthritis and autoimmune hemolytic anemia than did patients without these mutations[97,98]. An overproduction of immune cytokines or a better resistance to apoptosis in STAT3-mutated LGL cell population, including auto-reactive T cells, might explain this clinical association.

STAT5b mutations

Two different proven gain-of-function STAT5b mutations (Y665F and N642H) were found in 2% of STAT3 mutation-negative T-LGL patients with the N642H substitution being associated with an aggressive fatal disease course[99]. The same and new mutations were later encountered in 36% of T-PLL representing the highest frequency among all other mutated JAK-STAT components (JAK1, JAK3, IL-7Rα and γc) in the analyzed cohort[41]. STAT5b mutations are uncommon in T-ALL but their occurrence underlines the significance of the IL7R-JAK-STAT5 pathway in the pathogenesis of T-ALL[100]. Interestingly, while STAT5b mutant blasts were resistant to JAK inhibitors, the cells were highly sensitive to inhibitors of STAT5b target such as anti-apoptotic BCL-2 proteins[100].

STAT6 mutations

Identification of heterozygous point mutations in the DNA binding domain of STAT6 in 36% of PMBCL represented the first observation of recurrent STAT mutations in

human cancers[101]. Very recently, additional activating STAT6 substitutions were described in 11% of follicular lymphomas (FL) and were demonstrated to prolong residency of STAT6 in the nucleus resulting in a exaggerated transcriptional response to IL-4 highly present in the tumor microenvironment[102].

1.5 Loss of negative regulators

The activation of the JAK-STAT pathway is limited in time and amplitude by several regulatory mechanisms. For instance, constitutive phosphatases serve as quenchers of phosphotyrosines mediating activation and recruitment of downstream effectors. Additionally, inducible regulators of the suppressor of cytokine signaling (SOCS) family mediate ubiquitin-tagging for proteasomal degradation of the receptor complex, suppression of JAK catalytic activity or competition with STAT factors for docking sites on receptor. Loss-of-function alterations or epigenetic silencing affecting genes involved in the repression process are expected to result in more intense and sustained cytokine receptor signaling. Some illustrating examples will be documented in this section.

PTPN2 is a protein tyrosine phosphatase (PTP) able to dephosphorylate tyrosine residues located in the activation loop of JAK1 and JAK3[103]. Mono- or bi-allelic inactivation of PTPN2 through deletion of the entire gene locus was found in 6% of T-ALL cases[104], sometimes in co-occurrence with JAK1 activating mutations[104]. Downregulation of PTPN2 sensitizes to JAK1- but not JAK2-mediated transformation and reduces the sensitivity to JAK inhibitors of transformed cells[105]. Similarly, loss-of-function mutations of CD45, another JAK-regulating phosphatase encoded by the PTPRC gene, were detected in rare cases of T-ALL[106]. Remarkably, all CD45 mutations occurred in combination with activating mutations in IL-7Rα, JAK1, or LCK. Deletions or methylation of the PTPRK locus coding for a phosphatase that directly binds to and dephosphorylates STAT3, were recently reported in 55% of nasal NKTCL[107].

LNK is an adaptor protein that inhibits JAK2 activation downstream of TPO-R and EPO-R[108]. Mutations or deletions in the pleckstrin-homolgy (PH) domain of LNK

are reported in rare cases of MPN[109,110]. They occur either as an isolated event in chronic phase diseases[109] or concomitantly with JAK2 V617F mutation in patients that progress to AML[110].

The Casitas B-lineage lymphoma (Cbl) E3 ubiquitin ligase serves as negative regulator of proliferative signals by targeting a variety of tyrosine kinases and associated signaling components among which FLT3, c-kit, M-CSFR, JAK2, STAT5[111]. Homozygous loss-of-function mutations of Cbl lead to prolonged cytokine receptor complex activation and have been described in a small pourcentage of primary AML[112], as well as in classical and atypic MPN in which they have been associated with progression to secondary AML[113].

SOCS1 mutations were described in HL and PMBCL and were associated with nuclear phospho-STAT5 accumulation[114]. Reduced expression of SOCS1 and SOCS3, related to the hypermethylation of their promoter, has been described in different lymphoid hematological malignancies[115] among which MPN, in association[116] or not[117] with the JAK2V617F mutation.

Taken together, these observations underline the role played by deficiency of JAK-STAT negative regulation processes in the pathogenesis of diverse hematological neoplasms. Of note, such alterations can occur in concomitance with activating receptor or JAK mutations meaning that they are not mutually exclusive genetic events[104,106]. Hence, it is postulated that loss of suppression does not directly drive tumorigenesis, but rather contributes to the hyperactivation of the JAK-STAT pathway. Also, as tyrosine phosphatases are not JAK-restricted but possess multiple targets, their lack could exert a broader oncogenic impact on the intracellular signaling, beyond the JAK-STAT pathway. Finally, some of these alterations, such as PTPN2 deletions, reduce the sensitivity of transformed cells to JAK inhibitors indicating that their screening in patients could be relevant for the prediction of treatment response.

1.6 JAK inhibition in hematological malignancies

The oncogene addiction concept postulates that, despite the large amount of genetic lesions conveyed by a single cancer cell, some tumors rely on one single dominant oncogene for growth and survival, so that inhibition of this specific Achilles' heel is sufficient to halt the neoplastic phenotype[118]. At the clinical level, translation of the oncogene addiction model into the rational and effective design of targeted therapeutics against individual oncoproteins is best exemplified by the success story of Imatinib, an ATP-competitive tyrosine kinase inhibitor (TKI) of BCR-ABL that induced a dramatic shift in life expectation of CML patients[119]. The finding of aberrant JAK-STAT activation in a great majority of MPN and a significant portion of other hematological malignancies raised the hope for an Imatinib-like TKI therapy. The heterogeneity in the nature of the underlying genetic mechanisms can be overcome by exploiting the signaling bottleneck provided by the limited number of JAK kinases (Figure 4). However, it should be noted that activating mutations in STAT factors are relatively insensitive to JAK inhibitors.

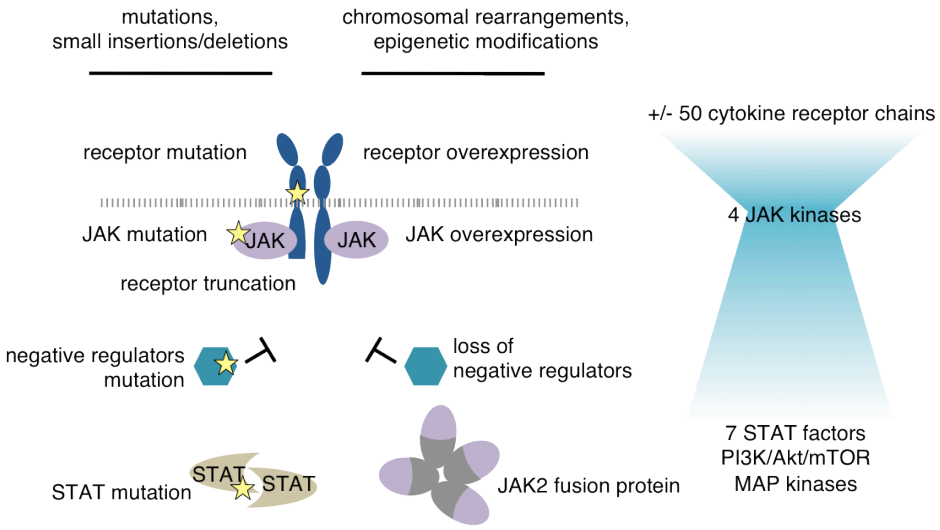


Figure 4. Mechanisms of constitutive JAK-STAT pathway activation in hematological malignancies. The molecular mechanisms underlying activation of the JAK-STAT pathway are depicted and can affect all the components of the cascade. They can result from small nucleotide sequence modifications or larger changes at the chromosomal level.

Ruxolitinib in myeloid malignancies

The discovery of the MPN JAK2 V617F mutation in 2005 has been rapidly transduced into the clinic. Not more than 6 years later, Ruxolitinib, a dual JAK1/2-specific ATP-competitive inhibitor, was US Food and Drug Administration (FDA)-approved for the treatment of intermediate- and high-risk MF, a disease for which allo-stem cell transplantation was the only curative option. In clinical trials, Ruxolitinib treatment yielded efficacy in controlling splenomegaly and alleviating MF-associated symptoms compared to placebo (COMFORT-I)[120] or best available therapy (BAT) (COMFORT-II)[121]. Surprisingly, MF patients benefit from Ruxolitinib therapy regardless of their JAK2 mutational status. Consistent with this unexpected observation, gene expression profiling data showed that MPN samples are characterized by a shared transcriptional signature of activated JAK2-STAT5 signaling, irrespective of disease phenotype or somatic genotype[122]. Subsequent identification of mutations in TPO-R and Calreticulin, which result in activation of the JAK2-STAT5 pathway as discussed above, provided an explanation for this

observation. The central role played by aberrant JAK-STAT pathway activation in MPN gave a rationale for the unrestricted efficacy of Ruxolitinib and raised the hope that mutation-positive and -negative PV, ET and MF patients could efficiently respond to JAK inhibitors.

However, Ruxolitinib exerted only limited salutary effect in terms of reversion of bone marrow fibrosis and reduction of allele burden, suggesting that the concentration required to kill neoplastic cells is impossible to reach in patients without inducing serious adverse events due to total inhibition of physiological cytokine signaling. Indeed, because of its lack of specificity towards JAK2 mutated forms, Ruxolitinib impedes physiological hematopoisesis leading to unwanted adverse events, the most frequently encountered being anemia and thrombocytopenia (incidence >20%)[123].

Alternatively, the MPN cells might not be JAK2 oncogene-addicted but the benefit observed on constitutional symptoms might be related to the alleviation of the proinflammatory tumor microenvironment rather than selective suppression of the disease clone[120]. The fact that Ruxolitinib also inhibits JAK1, an important player in innate and specific immune responses, might contribute to this anti-inflammatory activity. However, the drawback of this JAK1 inhibition is reduced control of silent infections and increased incidence of opportunistic diseases occasionally observed upon long-term administration[124-127].

Notwithstanding those side effects, the substantial bettering in life quality of MF patients encouraged the conduction of additional clinical trials, with the purpose to either enlarge Ruxolitinib indications or test other JAK2 inhibitors. Recently, a phase III clinical study aiming at evaluating the efficacy of Ruxolitinib as second-line treatment in PV patients intolerant to hydroxyurea came to the conclusion that JAK2 inhibition was superior to standard therapy in controlling hematocrit, reducing spleen size and improving PV-associated symptoms[128]. Ongoing phase III clinical trials in MF patients assess the efficacy and safety of less myelosuppressive novel JAK2 inhibitors such as Momelotinib (CYT387) versus Ruxolitinib (NCT01969838) and Pacritinib (SB1518) versus BAT (NCT01773187). Besides classical MPN, a

phase II trial currently estimates the potential of Ruxolitinib in CNL and atypical CML, where genetic activating alterations of CSF3R are encountered in a significant proportion of cases as described above and are associated with a poor outcome (NCT02092324). Ruxolitinib has already proved efficacy in a $CSF3R^{T618I}$ bone marrow transplant mouse model[129] and one case of $CSF3R^{T618I}$-positive atypical CML patient has been reported to experience significant clinical response to Ruxolitinib[130]. Further encouragement emanates from reports of two patients with PCM1-JAK2-positive chronic eosinophilic leukemia who achieved complete hematologic and cytogenic responses on Ruxolitinib[131,132], although, in two other described cases, rapid remission was only of limited duration and relapse occurred within 24 months[133].

Ruxolitinib in lymphoid malignancies

In pre-clinical experiments, Ruxolitinib treatment yielded a significant decrease of circulating blast counts in murine xenograft models of Ph-like B-ALL[134] and ETP-ALL[135], two recently described high-risk subtypes of ALL with aberrant activation of the JAK-STAT pathway for which novel therapies are needed. In the ETP-ALL model, the efficacy of treatment was independent of the presence of JAK-STAT mutations raising the possibility that the therapeutic potential of Ruxolitinib extends beyond the cases with JAK-STAT mutations[135]. As suggested for MPN, a JAK activation footprint may be more significant than the presence of a mutation for predicting response to therapy. This notion broadens greatly the numbers of patients that could potentially benefit from JAK inhibitors and will probably motivate the set up of future clinical trials in lymphoid malignancies. A recent clinical report described a unique case of refractory PMBCL with an activating JAK3 mutation (A573V) that experienced partial disease remission upon treatment with Tofacitinib, a JAK1/3-specific inhibitor, in combination with immuno-chemotherapy[136].

1.7 JAK inhibitor resistance in hematological malignancies

Albeit Ruxolitinib has proved effective in patients with MF, a fraction of them experienced treatment failure[137]. Besides discontinuation due to drug-related toxicity (such as early-onset cytopenias), Ruxolitinib treatment failure in MF manifests as primary refractoriness or secondary resistance. Primary refractoriness can be defined as the absence of onset of any or minimal clinical response (<35% reduction of spleen volume compared to baseline), whereas secondary resistance is indicated by the loss of previously confirmed clinical response (such as splenic relapse) or progression to leukemia[123]. In a cohort of 39 Ruxolitinib-treated MF patients, resistance was observed in 41% of cases, mostly as a late event (after median exposure of 1 year) and rarely as primary refractoriness (<10%). The same study identified a significant correlation between resistance and the absence of mutations in JAK2, MPL, TET2, and SRSF2 at diagnosis[138].

Based on clinical experiences with other TKI-therapies, it could be expected that resistant patients would have acquired mutations at the drug-binding site of the target kinase, as illustrated by the emergence BCR-ABL mutations in CML patients[119]. Yet, a dozen of different point mutations conferring resistance to Ruxolitinib were discovered using *in vitro* random mutagenesis screens of JAK2 and in vitro selection of spontaneous resistant cell lines[78,139-141]. However, up to now, no mutations in JAK2 could be detected in patients treated with Ruxolitinib, indicating that such mechanism is rarely involved in clinical resistance[138]. The tolerable dose of Ruxolitinib provides only a partial inhibition of the kinase, which might exert a therapeutic pressure insufficient for the selection of resistant mutants. Rather, the chronic exposure to suboptimal inhibitor concentrations might favor less drastic escape mechanisms. A reversible stage of so-called “inhibitor persistence” has been observed in Ruxolitinib-treated patients and in $JAK2^{V617F}$-transformed cell lines cultured with gradually increasing doses of Ruxolitinib *in vitro*[142]. Cells were allowed to persist despite JAK2 inhibition thanks to restoration of the JAK–STAT signaling via overexpression of JAK2, which facilitates its

heterodimeric transactivation with JAK1 or TYK2. Interestingly, inhibitors of HSP90, a chaperone known to stabilize JAK2, synergize with JAK2 inhibitors and overcome TKI persistence as well as genetic resistance[140,142,143]. A phase II study is currently evaluating the potency of a HSP90 inhibitor in MF and refractory PV/ET patients (NCT01668173).

1.8 Perspectives for the use of JAK inhibitors in hematological malignancies

At present, clinical data revealed that the targeted therapy concept prompted by the success story of Imatinib would be more complex to apply with JAK inhibitors. The incapacity of Ruxolitinib to achieve allele burden reduction is potentially due to the absence of strong JAK2 oncogene addiction of MPN cells as discussed before. Nevertheless, broader investigations are encouraged by the substantial benefit of JAK2 inhibition irrespective of mutational status in MPN patients, preclinical evidences for Ruxolitinib indication in additional malignancies (CNL, ETP-ALL, Ph-like ALL) and significant clinical response to JAK inhibitors in rare case reports ($CSF3R^{T618I}$-positive or PCM1-JAK2-positive MPNs and a $JAK3^{A573V}$-positive PMBCL).

A second hurdle resides within the lack of selectivity of the currently used inhibitors, which implies that the tolerable doses range within a narrow therapeutic window because of the collateral inhibition of other JAK members. Specific inhibitors for one of the four close-related JAK kinases could partially tackle this drawback but is challenging due to high similarity in the active site within the family. Ruxolitinib (and other JAK inhibitors under clinical evaluation) falls within the so-called type I family, which means that it precisely targets the well-conserved ATP-binding pocket of JAK1 and JAK2 in both active and inactive conformations[144,145]. By contrast, inhibitors that have a type II binding mode (such as Imatinib) specifically engage and stabilize inactive kinases by exploiting an additional less conserved allosteric site directly adjacent to the ATP binding pocket, providing another handle for tuning selectivity[144]. Type II inhibition of JAK2 with NVP-CHZ868 demonstrated a potent disease-modifying activity with significant reduction

of allele burden in different *in vivo* MPN models[146]. Alternatively, a covalent inhibitor exploiting a non-conserved cysteine residue in the active site of JAK3 has been shown to display strong target specificity[147]. Very recently, a new class of inhibitors were identified to bind to and lock the pseudokinase domain of TYK2 in a conformation that stabilizes the autoinhibitory interaction with the adjacent kinase domain, preventing its receptor-mediated activation[148]. Those JH2 stabilizers occupy a site analogous to the ATP-binding site of catalytic kinase domains by forming hydrogen bonds with non-conserved residues, presenting a novel approach in the design of highly selective inhibitors[148].

Paradoxically, increasing the specificity by developing type II, covalent or JH2 inhibitors could in turn result in a lower overall clinical benefit due the loss of collateral inhibition of cancer-related inflammation processes. In addition, one can speculate that more potent inhibitors would raise the selective pressure to a threshold that allows the emergence of tumor subclones harboring drug-resistant mutations, as observed with Imatinib therapy, stressing the need for alternative strategies.

MPN disorders stem from the constitutive activation of JAK2 at the apex of downstream signal propagation mediated by interconnected cascades of effectors (STAT3/5, PI3K/Akt/mTOR, MAPK) that synergistically act on cell proliferation[149]. The PI3K/Akt/mTOR pathway activation is required for JAK2 V617F-induced transformation of cells as well as JAK2 V617F-induced tumorigenesis in mice[150] and CD34+ cells derived from MPN patients showed increased levels of phospho-mTOR[151]. Therefore, simultaneous intervention at both arms of the JAK-STAT and PI3K signaling cascades became a rational intervention option. Two independent groups demonstrated that combining Ruxolitinib with a PI3K inhibitor synergistically inhibited proliferation of *in vitro* MPN cell models and reduced spleen weight in MPN preclinical models[152,153]. A similar synergistic action was also observed between mTOR and JAK2 inhibitors[154]. A translational phase I clinical study is currently evaluating the safety and maximal tolerated doses for the combination of Ruxolitinib and BKM120, a PI3K inhibitor, in MF patients (NCT01730248). In

addition to further clinical benefit, simultaneous blockade of the oncogenic signaling at multiple levels by combined therapy approaches (such as Ruxolinitib with PI3K inhibitors or HSP90 inhibitors) might avoid the emergence of resistance by distributing the selective pressure between distinct targets.

1.9 Conclusion

A promising future is awaiting JAK inhibitors in the therapeutic arsenal of hematological malignancies provided that appropriate indications are identified. Contrasting with the direct connection between Imatinib and BCR-ABL+ leukemias, the path to determining relevant applications for JAK inhibitors is more sinuous. First, the genetic alterations converging towards constitutive JAK activation are diverse and sometimes unexpected, such as the recently discovered calreticulin mutations. Furthermore, certain subtypes of ALL display an activated JAK transcriptional signature in the absence of known genetic abnormalities and respond to JAK inhibitors treatment in preclinical models, which makes the identification of suitable clinical indications even more complex. Second, once targetable candidate diseases are selected, their JAK oncogene-addiction remains to be clinically validated. Finally, the optimal therapeutic window is narrowed by the collateral inhibition of non-mutated JAKs, which represents a subtle balance between, on the one hand, adverse side effects due to impairments of physiological processes such as hematopoiesis or immunity and, on the other hand, beneficial contributions of alleviating cancer-related pro-inflammatory microenvironment. Therefore, it is very likely that JAK inhibitors combined to other targeted therapies will be more efficient in synergistically modifying the natural history of the disease, overcoming inhibitor persistence and preventing the emergence of resistant subclones.

2. Acquired resistance mechanisms against Tyrosine Kinase Inhibitors in cancer therapies

Oncogene addiction and targeted anti-cancer therapies

Development and survival of certain tumors rely on the activation of a specific pathway driven by a genetically altered or overexpressed oncoprotein, mostly receptor and non-receptor kinases, pinpointing a therapeutically exploitable "Achilles' heel"[155]. Indeed, the neoplasic phenotype of malignant cells is particularly susceptible to blockade of this single oncogene by small molecule inhibitors or monoclonal antibodies evoking a state of addiction, while inactivating the wild-type counterpart in normal tissue is relatively well tolerated, presenting a tumor-specific therapeutic window[118]. The molecular mechanism underlying oncogene addiction remains elusive but could involve a cellular reprogramming resulting in a strict dependence or a differential decay rate between the short-lived pro-survival and the long-lived pro-apoptotic signals emanating from the oncoprotein provoking a oncogenic shock following its acute inhibition[156].

Although switching-off the signaling cascade upon which cancer cells have become dependent can yield a temporary relief and even complete remission, its efficacy is invariably limited in time by acquired drug resistance phenomena highlighting the plasticity of tumors[157]. Insights into the various strategies implemented by tumor cells to circumvent inhibitory action of targeted therapies unveil recurrent themes, including alterations of the drug target, restoration of the oncogenic pathway and activation of compensatory signals via kinase switching or salutary growth factors production by the microenvironment. In addition, overexpression of ATP-binding cassette (ABC) transporters promoting drug efflux or inactivation of p53 mediating pro-apoptotic signaling have emerged as key factors contributing to multidrug resistance. It has been proposed that reduction of the intracellular small molecule inhibitor concentration or the failure to induce cell death might allow the progression of stronger adaptive mechanisms such as secondary mutations that are responsible for the replenishment of the tumor[158,159].

In this chapter, each type of evasion stratagem will be broached with particular emphasis on the molecular mechanisms of resistance to Tyrosine Kinase Inhibitors (TKI) with Imatinib as pioneering example.

Secondary point mutations in the drug target

Tyrosine kinases are highly amenable for the development of ATP-competitive small molecule drugs, however, owing to the conserved nature of the ATP-binding pocket, rationally designed TKI usually hit multiple targets at clinically used concentrations[160]. For instance, Imatinib, although being dedicated to inhibit ABL1, also binds with high affinity to c-Kit and platelet-derived growth factor receptor alpha (PDGFRα), which implies that it can treat tumors driven by the activation of anyone of those kinases, such as BCR-ABL positive CML and ALL [161], gastrointestinal stromal tumors (GIST)[162,163] and FIP1L1-PDGFRα-induced myeloproliferative diseases[164] respectively. However, Imatinib-treated patients frequently relapse predominantly due to the acquisition of point mutations in the kinase domain of the respective target. A great majority of them involve substitutions of the conserved threonine gatekeeper residue regulating access of TKIs to the ATP-binding cleft for a bulky amino acid[165], pointing to impaired drug binding by steric hindrance as the main cause of resistance (T315I in BCR-ABL, T670I in cKit and T674I in PDGRα)[166-169]. In addition to Imatinib, gatekeeper mutations appear as a common molecular mechanism of resistance against a variety of TKIs. Non-small cell lung cancer (NSCLC) patients frequently acquire the equivalent T790M mutation in Epidermal Growth Factor Receptor (EGFR) upon treatment with EGFR inhibitors[170,171]. *In vitro* screens for resistant mutations identified the F691I substitution at the gatekeeper position in FLT3[172,173] and analysis of engineered gatekeeper mutations in 4 other kinases (ABL1, PDGFRβ, SRC and FGFR1) demonstrate loss of sensitivity to selective inhibitors[174].

Besides the T315I mutation, at least 90 distinct point substitutions scattered throughout the kinase domain of ABL1 have been reported in Imatinib-resistant CML patients. One set of mutations, such as F359V, affects residues at contact points between the ABL kinase domain and the inhibitor, whereas a second

category, including the highly prevalent Y235F and E255K mutations, is postulated to destabilize an auto-inhibited conformation[175] to which Imatinib preferentially binds, shifting the equilibrium toward the active state that preclude drug binding[176,177]. This suggests that at least some BCR-ABL Imatinib-resistant mutations might act as intrinsic gain-of-function alterations of the fusion protein, independently of their effect on drug sensitivity. Indeed, several groups reported that the Y235F and E255K mutants displayed enhanced kinase activity[178,179] and transformation potency compared with native BCR-ABL[180]. Interestingly, this also applies for the gatekeeper mutations in several kinases among which EGFR as they were shown to stimulate kinase activity and promote malignant cell transformation[181-183]. This supports the hypothesis that acquired resistance results from the therapy-induced selection of pre-existing tumor subclones carrying drug-resistant mutations that were allowed to maintain in the population through increased cell fitness. Accordingly, sensitive allele-specific polymerase chain reaction–based analysis of therapy-naive CML[184-186] and NSLC[187,188] samples revealed the same mutation as the one detected at relapse, consistent with the selective model.

More potent second- (Dasatinib, Nilotinib and Bosutinib) and third-generation (Ponatinib) TKIs have been approved for treatment of CML and overcome nearly all Imatinib-insensitive mutants with the exception of the recalcitrant T315I that is only inhibited by Ponatinib[189]. However, those inhibitors still exert a therapeutic pressure that leads to the emergence of cells harboring novel (less numerous) resistant mutations mapping at contact points of the drug. Sequential switch from a TKI to another in relapsed CML patients may rescue clinical response as profiles of insensitive mutants are mostly specific to each drug, but carries the risk of selection for compound BCR-ABL mutations (i.e. 2 or more amino acid changes in a same molecule) with enhanced oncogenic potency[190,191]. This is of particular concern since clinically reported compound mutations pairing 2 key positions (most of the time including T315I) endows cells with a high resistance to TKIs, including Ponatinib[192]. Another drawback of sequential TKI use is illustrated by the I315M

substitution that evolved through a single nucleotide change from a baseline T315I mutation and confers a pan-TKI resistance[192].

Additional clinical illustrations of acquired kinase domain mutations upon TKI therapy are encountered in other kinase-driven malignancies such as FLT3 mutation-positive AML and BRUTON tyrosine kinase (BTK)-driven chronic lymphocytic leukemia (CLL). In contrast to the wide array of BCR-ABL resistance mutations, relatively few FLT3 and only one BTK resistant mutations have been identified[193]. Similarly to BCR-ABL, activating kinase domain mutations of FLT3 confer resistance by directly interfering with drug binding or inducing a switch into an active conformation[194]. Ibrutinib is a covalent inhibitor of BTK and replacement of the cysteine at the binding site (C481S) results in a protein that is only reversibly inhibited in relapsed CLL patients[195,196].

Amplification of the target

Another adaptation implemented by CML cells in order to reinstate BCR-ABL signaling despite inhibition involves intrachromosomal amplification of the genomic region encompassing BCR-ABL[166,197,198] or transcript overexpression[199] thereby altering the drug-target ratio. Similarly, increased copy number of EGFR and FLT3 genes has been observed in *in vitro* selected resistant cell lines[200,201] and in patients[202]. Finally, in JAK2-driven malignant cell lines, a state of so-called inhibitor persistence has been associated with increased JAK2 levels resulting from messenger RNA overexpression and protein stabilization[142].

Hiding of the target

Paradoxically to the previous paragraph, cells can evade targeted therapies by suppressing oncogene expression. Epidermal growth factor receptor 2 (HER2) is frequently upregulated serving as oncogenic driver in invasive breast cancers and can be targeted by a monoclonal antibody (Trastuzumab). Interaction of Trastuzumab with its antigen can be prevented by truncations of the extracellular

part of HER2 or epitope masking by higlhy glycosylated membrane-bound proteins[203].

Activating mutations in downstream effectors of the target

Some resistant cells restore oncogenic signaling by acquiring activating alterations in effectors downstream of the inhibited kinase resulting in a total independence on the primary oncogene. For instance, BTK inhibitor (Ibrutunib)-resistant CLL patients acquire gain-of-function mutations in Phospholipase C-γ2 (PLCγ2), which is directly activated by BTK in normal B-cell receptor signaling[195]. Computational evolutionary models predict that Ibrutinib-resistant mutants exist before initiation of therapy and have a small fitness advantage in the absence of treatment[204], as observed with BCR-ABL.

The PI3K/AKT/mTOR and RAS/RAF/MEK/MAPK cascades mediate intracellular response downstream of several tyrosine kinase receptors such as EGFR. Activating mutations in PI3KCα or BRAF are occasionally found in EGFR inhibitor-resistant NSCLC patients[202,205] and an activating mutation in MEK1 has been identified in one RAF inhibitor-resistant melanoma patient[206].

Activation of compensatory pathways

Inhibitor-treated cancer cells can compensate for the lack of oncogene signaling by switching their dependence towards functionally redundant kinases through the exploitation of pathway crosstalk and relief of negative feedback loops[207]. Numerous illustrations of alternative pathway activation in TKI-resistant cell lines and patients have been described in the literature. BCR-ABL addicted cells counteract inhibition by upregulating the activation of FYN, FAK[208] or PI3K[209] kinases. Likewise, resistance to EGFR inhibitors can be mediated by amplification of MET that drives the activation of the alternative kinase ERB3 in NSCLC [210,211] or by the relief of transcriptional PDGFRβ repression in glioblastomas[212]. BRAF-mutated melanoma cells are cross-resistant to BRAF selective inhibitors owing to a flexible switch among the three different RAF isoforms that rescues MAPK signaling[213]. The JAK-STAT, PI3K/AKT/mTOR and RAS/RAF/MEK/MAPK kinases

pathways are functionally interconnected effector cascades downstream of many interleukin receptors. Therefore, blockade of one signaling arm leads to the compensatory hyperactivation of one of the two others[214-216].

Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) is a critical dedifferentiation process by which cells lose adhesion and polarity, while gaining invasive and metastatic properties[217]. Furthermore, EMT contributes to TKI resistance and has been observed in inhibitor-insensitive hepatoma, lung and breast cancer cells[218,202,219]. The overexpression and constitutive activation of the receptor tyrosine kinase AXL (from the Greek word 'anexelekto', or 'uncontrolled') has been shown to orchestrate EMT leading to acquired TKI resistance and its pharmalogical inhibition restored sensitivity of cells to inhibitors[220,221]. The classification of tumors into epithelial or mesenchymal by assessing the expression AXL and downstream gene signature profile can serve as a predictive marker of response to targeted therapies[222,223].

Prosurvival signals from the microenvironment

Recent lines of evidence suggest that the tumor microenvironment promotes TKI resistance either by direct cell to cell contacts or via secretion of soluble growth factors[224]. For instance, co-culture with mesenchymal stromal cells (MSC) protected CML stem and progenitor cells from TKI-induced apoptosis through cellular adhesion[225]. Additional secreted factors might play a role since CML were shown to be less sensitive to BCR-ABL inhibitor in the presence of SCF[226]. Another study demonstrated that TKIs upregulated the expression of chemo-attractants, adhesion molecules, and prosurvival growth factors in MSCs. TKI-primed MSCs produced IL-7 that provided salutary survival signal to BCR-ABL+ ALL cells[227].

Perspectives for the use of TKIs in anti-cancer therapies

Altogether, these observations stress the unlikelihood for TKIs to represent a stand-alone successful long-term anti-cancer therapy considering the diversity of

resistance mechanisms resulting from intrinsic tumor plasticity and microenvironment interactions[157]. Nevertheless, rational drug combinations appear as promising therapeutic alternative options since the avenues for the development of drug resistance might be more limited[228] (Figure 5).

The most prevalent adaptation to ATP-competitive TKIs involves the acquisition of target mutations that modify the drug binding site or the overall conformation of the kinase domain and confer a fitness advantage in the absence of inhibitor. The emergence of such pre-existing subclones can be overcome by combining alternative inhibitors that differ in binding sites and thus have distinct spectra of insensitive mutants[229,230]. For instance, combination of BCR-ABL ATP-competitors with allosteric inhibitors suppressed the appearance of resistance mutations *in vitro* and displayed efficacy against the recalcitrant T315I mutant in a murine bone-marrow transplantation model[231].

Alternatively, some tumor cells restore oncogenic signaling through activating alterations in other components within the same cascade as illustrated with BRAF inhibitor-treated melanomas[232]. A phase III clinical trial demonstrated that cotreatment with inhibitors of BRAF and its downstream effector MEK, as compared with BRAF inhibitor alone, improved the rate of progression-free survival in previously untreated melanoma patients (NCT01584648)[233]. However, hitting a single signaling cascade at multiple points provides the tumor with a relatively simple escape route by activation of alternative compensatory pathways. This adaptation can be easily tackled by the use of multikinase inhibitors or co-administration of drugs targeting independent kinases such as EGFR and MET inhibitors in NSCLC, which proved efficient in overcoming EGFR-TKI resistance in pre-clinical models[234]. Additionally, prevention of EMT by AXL inhibitors and abolishment of stromal protective mechanisms with antagonists of growth factors receptors or adhesion molecules might substantially improve sustained clinical response to TKIs.

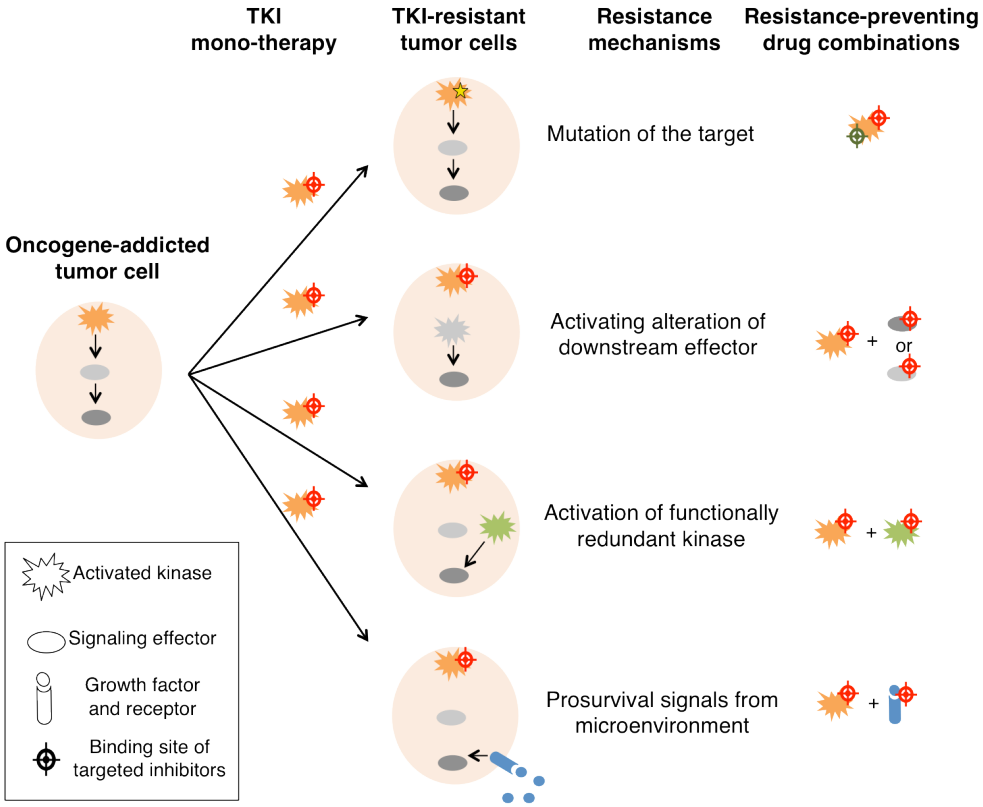


Figure 5. Main mechanisms of resistance against TKIs in tumor cells. Some tumor cells are addicted to the pro-survival signals emanating from a mutated or overexpressed tyrosine kinase, which renders them sensitive to the targeted blockade of this single oncogene. However, upon TKI treatment as monotherapy, resistant cells frequently emerged. The escape strategies implemented by resistant cells are diverse and can be overcome by specific rational drug combinations.

3. Mismatch repair and mutator phenotype of cancer cells

Mutations and cancers

Mutations are permanent and transmissible modifications of the genetic material arising from exposure to exogenous and endogenous mutagens, uncorrected replicative errors, inaccurate DNA repair and mistakes in chromosomal segregation during cell division. Commonly recognized forms of mutations encompass variations in the nucleotide sequence as well as abnormalities in the structure and number of chromosomes. Hereditable changes in the pattern of cytosine methylation and other DNA modifications without alteration of the primary DNA sequence are defined as “epimutations”[235].

In most cases, such modifications in the genetic information do not improve nor harm cellular functions but they occasionally manifest as an acquired aberrant phenotype such as anarchical proliferation or any one of the well-established hallmarks of cancer cells[236]. Malignant transformation stems from the sequential acquisition of diverse phenotypic abnormalities by accumulation in a single cell genome of multiple key mutations in tumor suppressors and proto-oncogenes.

Fortunately, the occurrence of such mutations is hampered by the extraordinary accuracy and efficacy with which cells normally replicate their genome and resolve DNA lesions (Figure 6). Indeed, human cells are equipped with 5 major DNA repair pathways, which correct specific types of mutations and DNA damages with a relative overlap and redundancy[237]. Three systems are specialized in the repair of single strand lesions (base excision repair, nucleotide excision repair and mismatch repair) while two others protect cells from chromosomal rearrangements by dealing with double strand breaks (non-homologous end joining and homologous recombination). In addition, DNA damage-sensors can trigger cell-cycle arrest or apoptosis above a critical threshold of unrepaired injuries. Altogether, the whole set of DNA repair effectors and damage sensors are termed "genome caretaker proteins". Finally, as ultimate safeguard, the limited number of mitoses undergone by somatic cells before entering a state of full differentiation or

senescence, represents a key mechanism for preventing the accumulation of mutations. The major contribution of this last point is buttressed by the strong positive correlation between the lifetime risk for a specific cancer type and the cumulative number of divisions undertaken by the self-renewing stem cells maintaining homeostasis of that particular tissue[238].

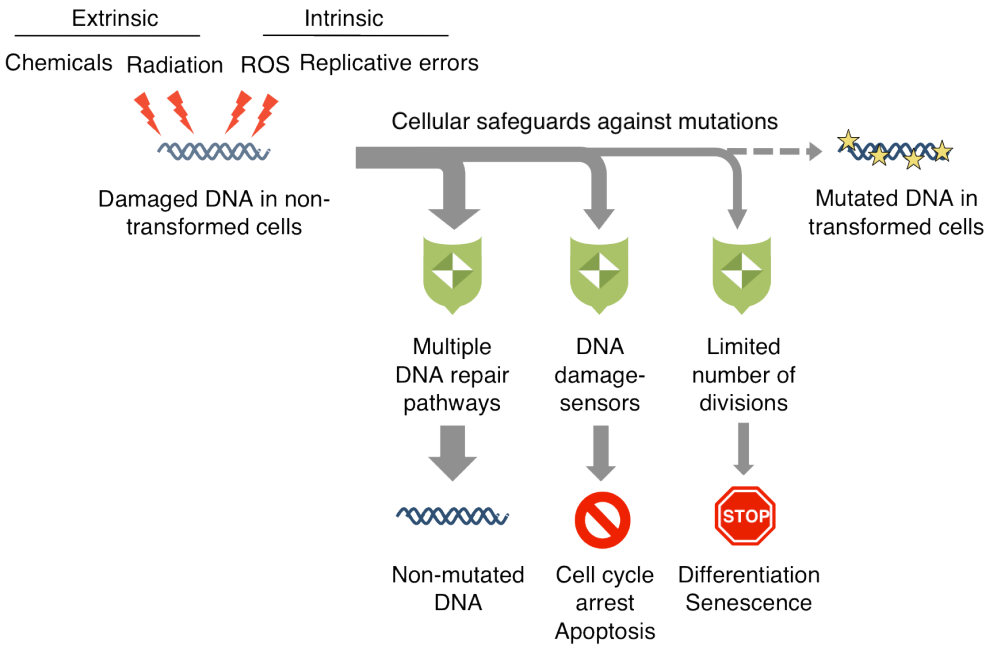


Figure 6. Cells possess several safeguards that prevent persistent mutations. The genome integrity is continuously challenged by extrinsic and intrinsic DNA damaging factors. Against this constant attack, cells dispose over escape routes leading to either DNA repair, mitosis arrest or cell death. In addition, the restricted number of divisions undergone by a single cell before differentiation or senescence limits the accumulation of mutations. Despite these safeguards, a small portion of DNA lesions persist and are converted into mutations that increase the risk for cancer development. (ROS) Reactive oxygen species.

Mutation rate in normal cells

Despite the armamentarium of cellular defense lines, a small fraction of damages evade and are converted into fixed mutations that are passed on to daughter cells when their occurrence is somatic or to all cells of the offspring when arising in the germ cells (egg or sperm). Germline mutations are the ultimate source of genetic

diversity within a population serving as a fuel for evolution of species. Historically, the rate of de novo mutations per generation has been estimated by indirect approaches such as analyzing the genome sequences at defined loci in close relatives or looking at phylogenetic distance between humans and chimpanzees[239]. The advent of next-generation sequencing (NGS) techniques provided a more direct and comprehensive method for comparing the entire genomes of parents-offspring trios. 3 independent pedigree studies came up with a consistent estimation of the single nucleotide variation (SNV) rate of 1.2 $x10^{-8}$ per base pair per generation, meaning that each zygote conveys about 75 de novo point mutations among the $6.4x10^{9}$ bases that constitute the human diploid genome[240-242]. However, those studies used DNA extracted from blood and the contribution of somatic mutations occurring early in development remained unclear[243].

The rate of de novo mutations per somatic cell division is more challenging to calculate and we still lack an accurate and unbiased estimation. Historically, due to technical limitations, the somatic mutation rate was rudimentarily measured in *in vitro* cultured cells based on the mutation frequency at sentinel loci, i.e. genes of which a wide range of single inactivating mutations are viable and manifest as a readily detectable phenotype. For instance, the X chromosome-linked hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus has been widely used because its invalidation confers an easily appreciable resistance to 6-thioguanine (6-TG). Practically, HPRT mutant frequencies were determined initially and at several time points while maintaining cells in a logarithmic growth. Mutation rate at the HPRT locus was obtained as the slope of the regression line plotting the 6-TG resistant frequency as a function of population doubling[244]. The HPRT sentinel method suffers from multiple shortcomings in estimating the global mutation rate of somatic cells within an individual. First, only inactivating genetic alterations are detected while neutral and activating mutations are not. Second, it seems likely that growing cells *in vitro* induces a plethora of genetic instabilities and estimation based on cell lines might not reflect the actual *in vivo* mutation rate. In addition, as it has become obvious that mutation frequency across the genome

is strongly influenced by replication timing, transcription levels and chromatin organization, measuring mutation rate on a small genomic portion provides a highly biased estimation [245,246].

The small scale issue associated with the sentinel method could be addressed by NGS of genomes from monozygotic twins that represent an attractive model for studying somatic mutations apart from germline ones, as any change between co-twins would arise from somatic events. 33 pairs of monozygotic twins were genotyped at 506,786 common single nucleotide polymorphism (SNP) sites across the genome in search for variations generated early in development that would result in heterozygous point mutations present in only one twin and absent in its co-twin[247]. Based on this study, the somatic point mutation rate was estimated as $6x10^{-8}$ per nucleotide per cell division meaning that somatic cells acquire 380 de novo mutations each time they replicate their genome.

Mutational load of cancer cells

Recent whole-genome sequencing of matched tumor-normal samples from a wide variety of cancer types revealed that the mutational load differs over several orders of magnitude. A single malignant cell genome conveys an averaged number of somatic lesions ranging from 1,000 in childhood brain tumors and acute leukemias, up to 100,000 in melanoma and lung cancers[248]. The ultimate purpose of these global sequencing projects was to create a comprehensive catalogue of significantly mutated cancer-associated genes that would represent potential targets for therapy[246]. Unexpectedly, the list of genes frequently mutated appeared to be disappointingly restrained (125 among 20,000 protein coding genes)[249] even within samples from the same tumor type highlighting the substantial inter-tumor heterogeneity and the stochastic nature of cancer evolution[250].

How cancer cells manage to acquire such a mutational complexity throughout tumor progression despite the relatively low mutation rate is under continuous debate. There exist two proposed scenarios for the accumulation of mutations in cancer cells, the power of natural selection and the mutator hypothesis. The first

model suggests that the mutational load results from the increased number of mitoses in highly proliferative cancer cells without an elevation of the intrinsic mutation rate. Alternatively, the second hypothesis postulates that acquisition of the large number of mutations present in cancer cells within a reasonable time requires an increase of the spontaneous mutation rate.

Natural selection for driver mutations

It is well established that cancer develops as the outcome of 2 connected processes occurring among cell populations within tissues of a multicellular organism, the continuous acquisition of random mutations by individual cells and the simultaneous natural selection acting on the resultant phenotypes[251]. Analogous to Darwinian evolution, the natural selection weeds out cells that have acquired deleterious mutations and fosters cells carrying alterations, defined as "driver mutations", that endow them with a proliferative or survival advantage over their neighbors. Most of the time, these competitions lead to the clonal expansion of cells with limited growth potential and manifest as benign proliferations. Occasionally, after repetitive rounds of selection, a single cell sequentially accumulates a sufficient set of advantageous genetic abnormalities, each of which exerts a small but positive effect on net cell growth that allows it to further progress into a malignant lesion. Epimutations can be subject to the same natural selection process, provided that they generate phenotypic changes for selective pressure to act on[251]. A classical tumor contains 2 to 8 of such rate-limiting mutations outnumbered by a overwhelming majority of passenger mutations, i.e. neutral alterations that do no influence cell fitness but expand because they coincidentally arose along with driver mutations[249].

Based on the most recent estimation of the spontaneous somatic mutation rate ($6x10^{-8}$/bp/division) and the rationalistic assumption of maximum 100 mitoses since cancer initiation, tumor genomes might bear about 40,000 somatic mutations. Accordingly, the scenario where tumor development is only driven by the strength of natural selection for cells that stochastically acquired driver mutations at a

normal rate is compatible with the greatest part of cancer cases. However, a subset of genomes from endometrial, stomach, colorectal, lung and skin cancers (representing 3.6% of all analyzed cancers) displayed more than 100,000 somatic mutations, a phenotype referred to as "hypermutation"[252]. In hypermutated lung cancers and melanomas, mutational burden could be explained by an established chronic exposure to particular mutagens, tobacco smoke and ultra-violet light respectively. In the remaining hypermutated cases, an increase of the intrinsic mutation rate must be required in order to reach such a heavy mutational load.

Mutator phenotype and increased likelihood of acquiring driver mutations

As mutations occur more or less randomly across the genome, some of them affect genes encoding caretaker proteins resulting in a reduced genome stability or "mutator phenotype". Mutator cells have an elevated intrinsic mutation rate that increases the likelihood to acquire advantageous alterations required to overcome each selective bottleneck in cancer evolution[253]. Consequently, across multiple rounds of natural selections for driver mutants, there is a simultaneous progressive enrichment of inactivating mutations in caretaker genes that will further generate heterogeneity and fuel tumorigenesis[254]. This concept is supported by studies in E.coli demonstrating that, within a population, the proportion of bacteria expressing a mutator phenotype is greatly increased after growth on restricted medium and can be further enhanced by sequentially submitting the surviving colonies to additional selective pressures[255]. Both environmental selective pressure and genetic instability are likely to contribute to cancer progression and may be tightly linked and interdependent as illustrated on figure 7.

The involvement of a mutator phenotype in human cancers emanated from the discovery of the etiological factors causing cancer-predisposing hereditary syndromes with marked genetic instability. Indeed, inherited cancer syndromes frequently involve loss-of-function mutations in genes involved in DNA repair. This was first highlighted in Xeroderma Pigmentosum, where patients develop multiple skin cancers during childhood due to an inherited defect in one component of the

nucleotide excision repair (NER) pathway specialized in the resolution of helix-distorting DNA lesions such as photo-induced pyrimidine dimers[256]. Later on, impairments of the mismatch repair (MMR) system, which removes biosynthetic errors arising in the newly replicated strand during genome duplication, were found to underlie hereditary non-polyposis colon cancer (HNPCC) syndrome[257].

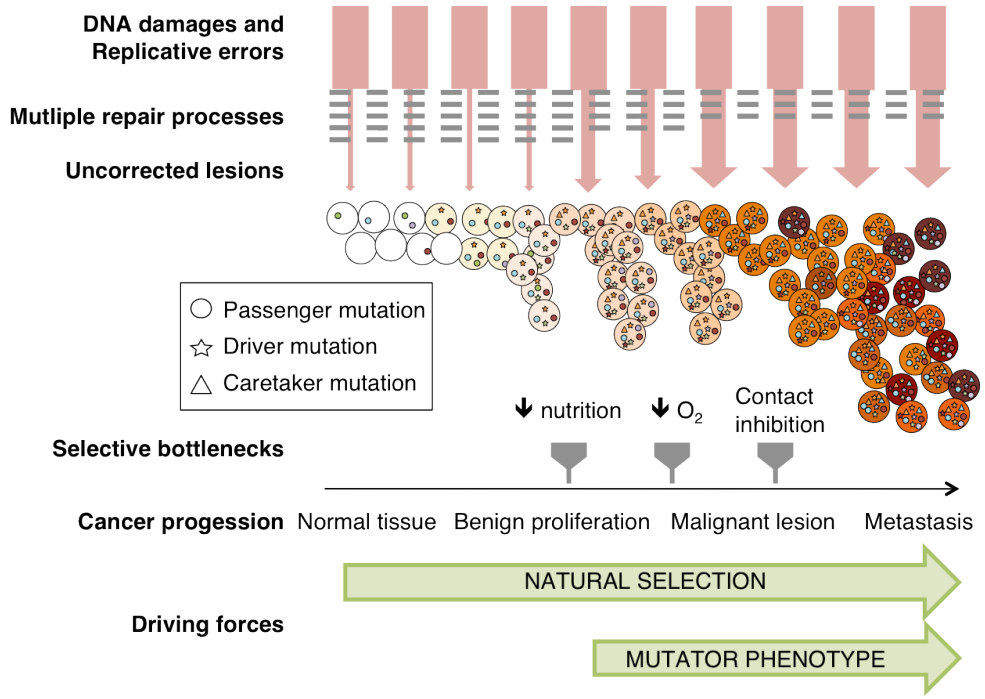


Figure 7. Cancer progression is driven by natural selection and mutator phenotype. In normal tissue, cells accumulate mutations as a result of DNA lesions that escaped from the multiple repair processes. Natural selection fosters the expansion of cells that have acquired advantageous phenotypes conferred by driver mutations. With each selective bottleneck related to tumor growth (for example: reduced nutrition, hypoxia, cell-cell contact inhibition, anti-tumoral immunity), malignant cells with increasing numbers of driver mutations will outgrow. Cells that acquired by chance a loss-of-function mutation in caretaker genes will accumulate more mutations and have a higher likelihood to overcome selective pressure. Increased genetic instability, referred to as mutator phenotype, will further generate additional mutations resulting in genetically distinct cell variants within the tumor population.

Postreplicative mismatches

DNA replication fidelity is guaranteed by two components highly conserved among species that both contribute to mutation avoidance. In mammalians, the first safeguard resides within the intrinsic accuracy of nuclear DNA polymerases-ε and -δ (encoded by the genes POLE and POLD1) mediating synthesis of leading and lagging strands respectively. Replicative polymerases possess a high selectivity for Watson-Crick base pairing and err only once every 10^4 nucleotides, in the presence of a balanced dNTP pool. Moreover, replicative polymerases possess a 3'-5'-exonuclease activity that preferentially excises non-complementary nucleotides prior to the incorporation of the next base, lowering the error rate by 1 order of magnitude (10^{-5}). Besides the 2 major replicases pol-ε and -δ that perform the bulk of the work, 6 additional exonuclease-deficient DNA polymerases (pol-ζ, -η, -ι, -κ, -ν, -θ) contribute to genome duplication by incorporating nucleotides opposite to DNA lesions that otherwise impede progression of pol-ε and -δ, hence the term "translesion polymerases"[258].

Mismatches generated during DNA chain elongation that persist once the replication fork has passed must be corrected before the next cell division in order to avoid mutation in 50% of progeny. They are handled through a second-line defense provided by the MMR machinery, further improving the overall fidelity of genome duplication by 2-3 orders of magnitude.

Overview of the mismatch repair system

MMR is a multicomponent system that requires the coordinated action of at least 10 proteins in order to achieve efficient mismatch recognition, strand discrimination, excision of the error-containing nascent DNA tract and replacement by the correct sequence (Figure 8)[259,260]. MMR process is initiated by dimers of the bacterial MutS homologs (MSH) that cover double stranded DNA and scan it in a search for helix distortions caused by non-complementary base pairs. There exist 3 mammalian MSH proteins that associate as 2 different heterodimers, either MSH2-MSH6 (MutSα) or MSH2-MSH3 (MutSβ) recognizing respectively small

mismatched or misinserted bases (1-2 nucleotides) and larger insertion-deletion loops (IDL). Upon DNA heteroduplex engagement, the MutS dimer undergoes a ADP-ATP exchange-driven conformational change into a sliding clamp and recruits a second heterodimer composed of MutL homologs (MLH). 4 mammalian homologs for MutL protein have been identified (MLH-1, -3 and post-meiotic segregation (PMS)-1,-2) that assemble as 3 distinct heterodimers, each of which comprising a MLH1 subunit. The MLH1-PMS2 complex (referred to as MutLα) is the main regulator of downstream steps of repair. The 2 other MLH heterodimers, MutLβ (MLH1-PMS1) and MutLγ (MLH1-MLH3), participate to a lesser extent to MMR and partially compensate for the lack of MutLα *in vitro*, but fulfill other functions like chromosome segregation and meiotic recombinations[261].

At this step of MMR process, the mismatch recognition factors face the issue of strand discrimination. Because the correct genetic information resides within the template strand, excision must be directed to the error-containing nascent strand. Distinction between both is mediated via discontinuities in the newly synthesized strand providing loading sites for exonuclease-1 (EXO1) and requires the interaction of MSH-MLH dimers with proteins from the replication machinery, Replication factor C (RFC) and Proliferating cell nuclear antigen (PCNA) associated respectively with the 5'- and 3'-termini of the nascent DNA strand. The MutS-MutL complex can diffuse along DNA in both directions and will unavoidably encounter a strand end. Concerning the lagging strand, gaps between 2 Okazaki fragments endow EXO1 with ample initiation sites for hydrolyzing phosphodiester backbone with a 5'-3' polarity. By contrast, incisions in the continuous leading strand are generated by the latent endonuclease activity of MutLα, which is potentiated upon association with PCNA and RFC[262,263]. Once its endonuclease function is stimulated, MutLα introduces random single strand breaks including nicks 5' distal to the mismatch that will serve as starting point for the 5'-3' directional excision of the erroneous DNA tract. Replication protein A (RPA) protects single stranded DNA generated during excision and facilitates resynthesis by pol-δ followed by nick ligation mediated by DNA ligase-I[264].

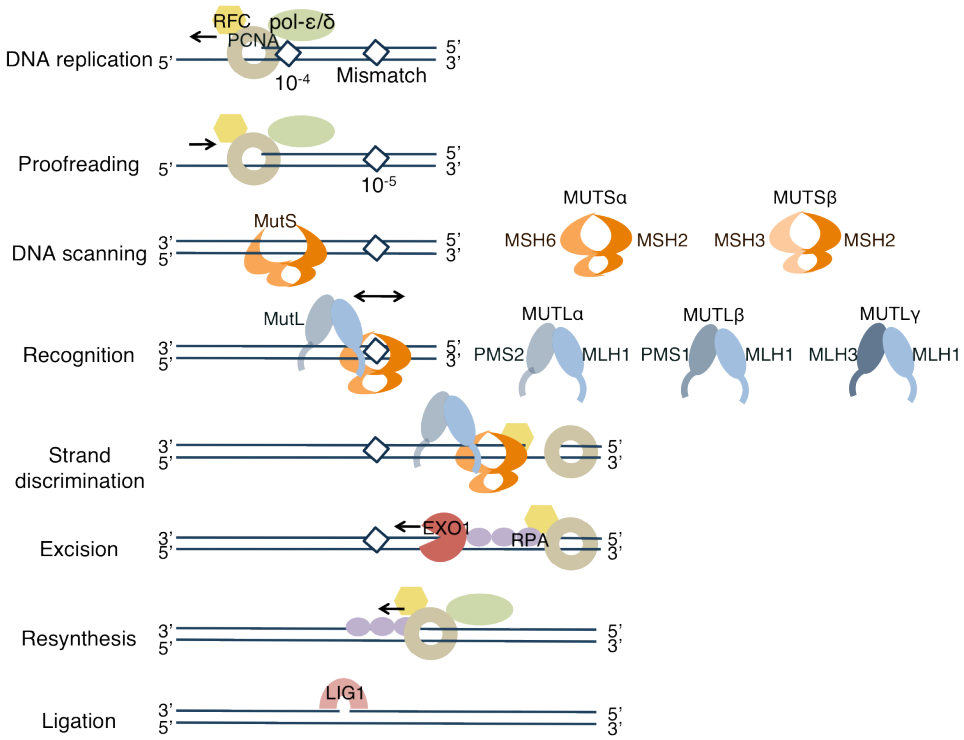


Figure 8. Resolution of polymerase errors by the proofreading activity and the mismatch repair system. Mismatches that escape proofreading activity of replicative polymerases are recognized by one of the 2 MutS dimers (MutSα/β). Once bound to a mismatch, MutS recruits a second heterodimer (MutLα/β/γ). Together they form a sliding clamp that diffuses along DNA in search for strand discrimination signal provided by DNA termini associated with replication factor C (RFC) and proliferating cell nuclear antigen (PCNA). Strand discontinuities serve as loading sites for the 5'-3' exonuclease1 (EXO1) that excises the error-containing DNA tract generating single stranded-DNA protected by replication protein A (RPA). The missing piece is resynthetized by polymerase-δ and both ends are ligated by DNA-ligase1.

Modulation of MMR

Since MMR is an immediate post-replicative correction mechanism, its activity is unsurprisingly coordinated in time and space along with the cell cycle. MMR genes reach their highest expression level during the DNA synthesis S phase[265]. In addition, the epigenetic histone marker H3K36me3, abundant during G1 and S phases, directly interacts with MSH6 thereby ensuring that MutSα is enriched on

chromatin before mispairs are introduced during duplication[266]. MMR process seems to be more effective in early replicating regions compared to late replicating heterochromatin which generates somatic mutation rate variations across the human genome[267]. Differences in accessibility of the repair machinery to DNA and the time available for correction before the next round of replication might contribute to this phenomenon. As genes fulfilling essential cellular functions map into early-replicating regions, it is conceivable that enhanced MMR in euchromatin is a beneficial trait protecting against cell survival-incompatible DNA lesions that has been selected for during evolution[267].

Efficacy of MMR can be influenced by additional intrinsic and extrinsic factors, some of them are related to tumorigenesis. As such, imbalanced dNTP pool decreases the fidelity of replicative polymerases[268]. MicroRNA (miR)-155 and miR-21 down-regulate the expression of MLH1, MSH2 and MSH6 [269-271]. MLH1 is transcriptionally repressed under hypoxic stress[272,273]. EGFR signaling mediates the phosphorylation of PCNA thereby inhibiting its interaction with MMR proteins[274]. Finally, acquired MMR deficiency involving epigenetic silencing of MLH1 occurs naturally in the ageing process of hematopoietic stem and progenitor cells, which might partially explain why hematological malignancies arise at a higher rate in older individuals[275].

MMR deficiency in familial cancers

As already mentioned, mutations in genes encoding proteins involved in the MMR system are highly prevalent (65%) among families with HNPCC also known as Lynch syndrome, which is characterized by a dominantly inherited predisposition to a spectrum of cancers, primarily of colorectal and endometrial origins[257]. HNPCC individuals classically inherit a heterozygous loss-of-function alteration in genes encoding one the 7 mismatch recognition proteins that endows them with a high susceptibility to tumor development following somatic inactivation of the remaining allele by loss of heterozygosity. Mutations are highly prevalent in MSH2 and MLH1 (50% of cases), less frequent in MSH6 (10%) and PMS2 (5%) while being very rare

in MSH3, MLH3 and PMS1, reflecting the key role of the 2 obligatory components of MutS and MutL heterodimers and the functional redundancy between other MSH and MLH factors[276]. The majority of the genetic MLH1 and MSH2 alterations are frameshift and nonsense mutations resulting in truncated proteins unable to interact with partners[277]. MLH1 can also be affected by missense substitutions of key residues. MLH1 and MSH2 mutations carriers have a cumulative risk for colorectal cancer at the age of 70 of approximately 40%[278].

A minority of families meeting the HNPCC clinical criteria but without alteration in the primary coding sequences of MMR proteins were shown to convey soma-wide mono-allelic methylation of the MLH1 or MSH2 locus, as an alternative cause of Lynch syndrome. Concerning the MLH1 gene, the molecular alterations underlying such epimutations are diverse (SNV in promoter, deletions of the first exons or larger rearrangements of the MLH1 flanking region)[279-281]. By contrast, MSH2 epimutation are mainly caused by recurrent deletions in the 3' region of the upstream adjacent gene (EPCAM) encompassing its poly-adenylation signal. This results in a transcriptional read-through from EPCAM into the MSH2 locus and inactivation of the MSH2 promoter by methylation in tissue where EPCAM is expressed[282].

Germline mutations in the EXO1 gene identified in otherwise mutation-negative HNPCC patients were shown to encode mutants with reduced nuclease activity[283] or impaired interaction with MSH2[284]. However, their implication in HNPCC is still debated as some EXO1 variants occur naturally in healthy population[285].

Some individuals with dominantly inherited susceptibility to colorectal adenomas and carcinomas, resembling HNPCC, but in which mutations of known predisposing genes have been excluded, were found to carry an heterozygous germline missense mutation in POLD1 or POLE genes encoding the catalytic subunits of polymerases ε and δ. Mutations map to the exonuclease domain and are predicted to impair the proofreading function of polymerases assuring the first safeguard against post-replicative mismatches[286].

Bi-allelic MMR-deficiency syndrome

Rare individuals that inherit 2 mutations in both alleles of one MMR gene (MLH1, MSH2, PMS2, MSH6) present with childhood brain tumors and hematological malignancies (predominantly T-cell leukemias and lymphomas) in addition to early-onset of colon cancer, a syndrome referred to as bi-allelic MMR-deficiency (b-MMRD)[287]. A recent whole-genome sequencing study showed that a subset of brain tumors from bMMRD patients displayed an ultra-hypermutated phenotype (> 250 point mutations per Mb), largely surpassing the mutational load of a reference dataset of 7,000 pediatric and adult cancer genomes[288]. Interestingly, non-neoplasic cells from these patients have a similar number of variants compared to control individuals suggesting that cancer developed further to a second genetic hit. This latter turned out to be an early acquired somatic loss-of-proofreading mutation in POLE or POLD1, which results in a total ablation of post-replicative errors repair. As a consequence, malignant cells rapidly accumulate a large excess of de novo mutations each time they replicate, reaching but not exceeding the upper limit of 20,000 exonic mutations. This number represents the critical threshold for tolerable mutational load above which damage of additional genes becomes incompatible with cancer cell survival because essential cellular functions would be impaired[288].

MMR deficiency in sporadic cancers

Besides familial cancers, MMR defects occur in a significant proportion (12-20%) of sporadic counterparts of the HNPCC-spectrum tumors, namely cancers of the gastro-intestinal, gynecologic and urologic tracts, principally due to bi-allelic MLH1 methylation and more rarely to mutations in MMR genes[289-291]. Recently, comprehensive molecular characterization of colorectal and endometrial cancers classified tumors based on mutation frequencies into hypermutated (>12 mutations/megabase) and ultra-hyper mutated (>50 mutations/megabase)[292,293]. Hypermutated tumors harbored the MLH1 methylation profile usually observed in sporadic HNPCC-spectrum tumors while ultra-hypermethylated tumors conveyed

mutations in the exonuclease domain of POLE combined or not with genetic abnormalities of other MMR factors, similarly to bMMRD.

The increased risk for childhood leukemias and lymphomas associated with bMMRD syndrome suggests that alterations in MMR genes could underlie sporadic hematological malignancies. Furthermore, knocking-out MSH2 or MLH1 in mice causes an increased incidence of early-onset lymphomas (predominantly of the thymus)[294,295]. Historically, first evidences for involvement of compromised MMR in human sporadic blood cancers have been provided by means of leukemia and lymphoma cell lines in which inability to repair mismatches *in vitro* correlated with loss of expression or mutations in MMR genes[296,297]. Those observations are now confirmed in patients. MLH1 expresssion is epigenetically silenced in 24% and 63%

of AML and NKCL patient samples[298,299]. Cells from adult T-cell leukemia (ATL) patients display reduced or loss of expression of at least two MMR proteins[300].

Interestingly, some sporadic tumors display features of MMR deficiency but no detectable genetic or epigenetic alterations in known predisposition genes, indicating that there exist other ways for a malignant cell to downregulate the repair fidelity of mismatches. Recently, two MMR-deficient cancer cell lines were shown to convey inactivating mutations in the SETD2 gene coding for the histone modifying enzyme that catalyses the trimethylation at the lysine 36 of histone 3 generating the epigenetic marker H3K36me3 needed for the recruitment of MutSα to chromatin[266]. Conversely, cells that artificially overexpress KDM4C, the enzyme that demethylates H3K36me3, are defective in MMR function[301]. At present, such genetic alterations affecting the epigenetic machinery were only observed *in vitro* but represent serious alternative candidates to explain MMR deficiency of cancer cells devoid of "classical" lesions. Finally, the expression of miR-155 is inversely correlated with MLH1 and MSH2 protein amounts in colorectal cancer tissues and a number of colon tumors with unknown cause of MMR inactivation overexpress MiR-155[270]. Table 1 gives a general outlook of the catalogue of the cancer-related genetic alterations affecting the MMR system.

Gene	Type of genetic alterations		Diseases
MSH2	Inactivating mutation	Hereditary	HNPCC/bMMRD
	Epimutation	Hereditary	HNPCC
MSH6	Inactivating mutation	Hereditary	HNPCC/bMMRD
MSH3	Inactivating mutation	Hereditary	HNPCC
MLH1	Inactivating mutation	Hereditary	HNPCC/bMMRD
	Epimutation	Hereditary	HNPCC
	Bi-allelic methylation	Sporadic	HNPC-spectrum tumors leukemia/lymphoma
PMS2	Inactivating mutation	Hereditary	HNPCC/bMMRD
PMS1	Inactivating mutation	Hereditary	HNPCC
MLH3	Inactivating mutation	Hereditary	HNPCC
EXO1	Inactivating mutation	Hereditary	HNPCC
POLE/ POLD1	Loss of proofreading mutation	Hereditary	Colorectal adenomas and carcinomas
		Sporadic	Ultra-hypermutated colon and endometrial cancers
			Ultra-hypermutated brain tumors in bMMRD
SETD2	Inactivating mutation		In vitro cell lines
KDM4	Overexpression		In vitro cell lines
miR-155	Overexpression	Sporadic	colorectal cancer

Table 1. Genetic alterations affecting the MMR system in human malignancies and cell lines

Mutational pattern of MMR-deficient tumors

While most cancers typically harbor gross chromosomal rearrangements and aneuploidy, MMR-deficient tumors tend to be diploid and exhibit a normal rate of chromosomal alterations[302]. Genetic instability in cancer with compromised MMR almost invariably manifests as length variations in genomic regions composed of repeated short nucleotide tracks called microsatellites, a phenotype referred to as microsatellite instability (MSI). Owing to their repetitive nature, microsatellites are particularly susceptible hotspots for slippage errors occurring by mispairing

between template and newly synthesized strands resulting in small IDL that are not properly corrected in the absence of MMR and give rise to differences in the number of repeats.

In addition to serve as a widely used diagnostic marker indicating that the tumor has developed as a consequence of MMR deficiency, MSI actively drives tumorigenesis by promoting frameshift mutations in microsatellites within the coding sequence of known tumor-suppressors. Accordingly, in MSI+ colorectal cancers, the TGFβ receptor II (TGFBR2) and the pro-apoptotic protein BAX are recurrently inactivated by slippage-induced insertions/deletions within polynucleotide tracts present in their open reading frames[303,304]. Interestingly, MSH3 and MSH6 genes also contain mononucleotide repeats that are frequently subject to inactivating frameshift mutations, further increasing the genetic instability of MSI+ cells[305]. The drawback of this increased mutability is that it boosts at the same time immunogenicity by generating tumor-specific antigens in sufficient amount to elicit T-cell responses[306]. Nevertheless, MSI+ tumors manage to evade anti-tumoral immunity by acquiring frameshift mutations in genes encoding key actors of antigen presentation, predominantly truncations of the light chain of the HLA-I complex (B2M) but also proteins of the antigen processing machinery such as the transporters TAP1 and TAP2[307-310].

Besides frameshift mutations in microsatellites, deficits in MMR function also enhance the rate of spontaneous and carcinogen-induced single base substitutions in MMR-deficient mice and cell lines, which could be corrected by complementation for the missing MMR gene[311-313]. Analysis of exome-sequencing data of MSI+ colon tumors generated a list of missense mutation hotspots in 33 genes and led to the identification of novel oncogenes[314].

Figure 9 proposes a graphic schematization for the evolution of the mutational load as a function of time in hereditary syndromes and sporadic cancers related to MMR-deficiency. Key inherited and acquired genetic alterations are indicated, some of them having a slope-modifying effect because they increase intrinsic mutation rate leading to hypermutated and ultra-hypermutated tumors.

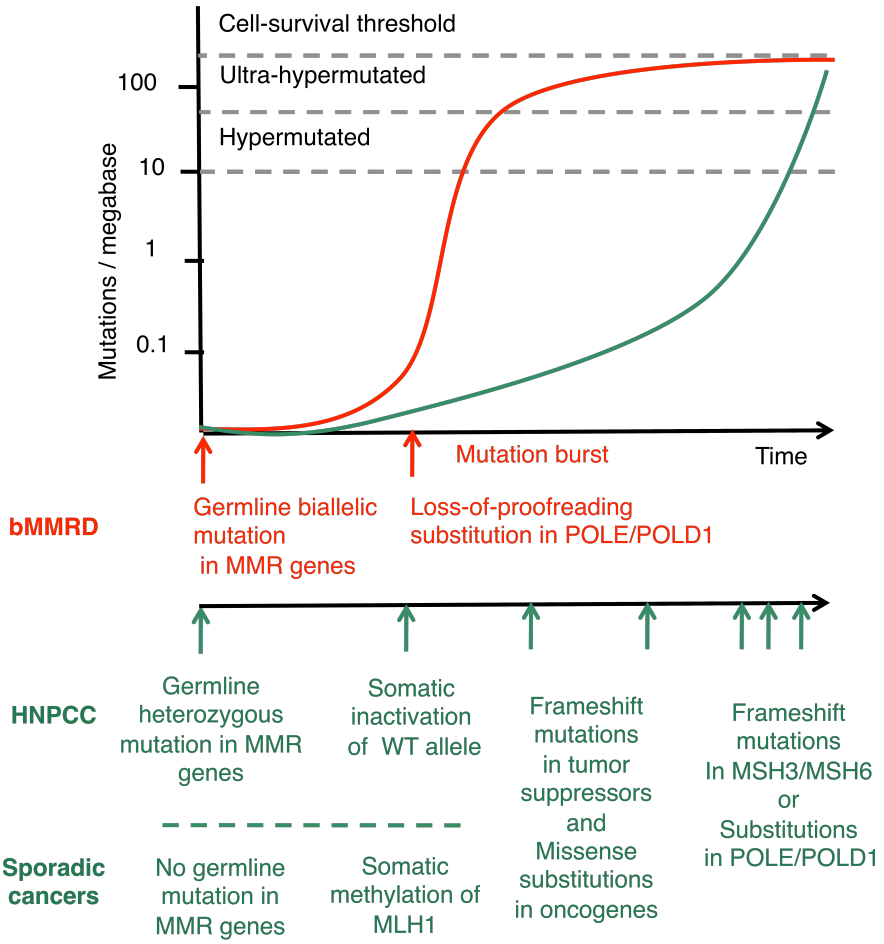


Figure 9. Evolution of the mutational load in MMR-deficient tumors. In biallelic MMR deficiency syndrome (bMMRD), individuals inherit bi-allelic mutations in one MMR gene. Upon early acquisition of mutation in one gene encoding replicative polymerases (POLE or POLD1), cells accumulate random mutations in a rapid burst reaching a plateau at the cell survival compatible threshold (20,000 exonic mutations). As a result, bMMRD patients present with very early cancer onset during childhood, mostly brain tumors and hematological malignancies. By contrast, in hereditary non-polyposis colon cancer (HNPCC), individuals inherit a germline heterozygous (epi)mutation in one MMR gene and cells accumulate mutations only after somatic inactivation of the remaining WT allele that occurs later in life. Alternatively, MMR-deficient tumors can develop after somatic bi-allelic methylation of MLH1 in individuals without mutation in MMR genes at birth. In both cases, cells accumulate point substitutions but preferentially frameshift mutations in genes containing microsatellites including tumor suppressors and MMR genes (MSH3 and MSH6) resulting in an even further increased mutation rate leading to hypermutated cancers (mutational load between 10 and 50 mutations/megabase). Inactivating mutations of POLE/POLD1 can also occur leading to ultra-hypermutated tumors (50-200 mutations/megabase).

In conclusion, the mutator phenotype characteristic of MMR deficiency endows tumors with a specific spectrum of genetic abnormalities irrespective of hereditary and sporadic origin, namely frameshift deletions/insertions in tumor suppressor genes and missense mutations in oncogenes[315]. Interestingly, the ensuing mutational landscape is distinct from MMR-proficient counterparts [316], indicating that MSI+ and MSI- tumors progress through different carcinogenic genetic events and might therefore represent variant clinicopathological entities. For instance, MSI+ colorectal cancers have fewer APC, KRAS and TP53 mutations while displaying higher numbers of BRAF and TGF-β pathway-related alterations in comparison with MSI- tumors.

Non-canonical functions of MMR proteins

Additionally to correction of postreplicative errors during the S phase of mitosis, MMR proteins also take part in the cellular response to specific DNA damages and in the processing of modified bases[317]. Although base excision repair (BER) and nucleotide excision repair (NER) are the two main pathways believed to orchestrate the removal of modified nucleotides, MutSα has been shown to interact with oxidized, alkylated or crosslinked bases[318,319]. 2 models have been proposed to describe the mechanism by which MMR proteins activate signaling cascade leading to prolonged G2 arrest and eventual cell death [320]. In the first model, MMR proteins bind DNA lesions and initiate futile repetitive cycles of repair that will be aborted after several unsuccessful attempts leaving a gap opposite to the damaged base[321]. These single-stranded breaks persist until the next round of replication when cell cycle checkpoint proteins recognize them as aberrant DNA structures and provoke mitosis arrest or cell killing. The alternative scenario is in favor of an active role of MMR factors by directly recruiting the Ataxia telangiectasia-mutated (ATM) or ATM- and Rad3-related (ATR) protein kinases which in turn activate downstream substrates that will relay cell-cycle arrest and apoptosis signaling via different pathways depending on the type and level of DNA damages.

In conclusion, MMR deficiency contributes to tumor development by exerting a dual deleterious effect: on the one hand, increased mutation rate due to persistence of replicative errors as well as modified bases and, on the other hand, defective apoptosis in response to DNA damage. Accordingly, studies of mice carrying mutations that selectively inactivate the mismatch repair activity of MSH2 but preserve its damage-response function indicate that the increased mutation rate in MMR-deficient cells is sufficient to drive tumorigenesis but defective apoptosis can further accelerate it[322].

Clinical implications of MMR deficiency in tumors

One major clinical implication of MMR pathway playing a role in DNA damage signaling resides in the fact that many of the anti-cancer chemotherapeutics are DNA-damaging agents. Consequently, cancerous cells lacking a functional MMR pathway tolerate the presence of injured DNA resulting in primary refractoriness to a wide range of clinically used genotoxic molecules such as 6-thioguanine, alkylating agents and cisplatin derivatives [323-328]. Moreover, initially MMR-proficient ovarian cancer cells can acquire a MMR-deficient phenotype by MLH1 promoter silencing upon exposure to chemotherapeutic drugs *in vitro*[329,330]. In line with this, hypomethylating agents like 5'-AZA-2'deoxyCytidine have been shown to resensitize cisplatin-resistant MLH1-silenced ovarian and colon cancer cells *in vitro* and *in vivo*[331,332]. All these experimental data are supported by consistent clinical observations. Loss of MMR function was significantly more prevalent in AML patients with chemotherapy-relapsed diseases compared with newly diagnosed patients[333]. Furthermore, MMR defects are widespread among secondary cancers that arise after successful chemotherapy for a primary malignancy[334,335].

Besides primary tolerance to classical DNA damaging anti-cancer treatment, MMR deficiency in tumors is postulated to also affect the efficacy of targeted therapies. Indeed, as describe earlier, the absence of efficient MMR system confers an increased mutation rate or mutator phenotype. The mutator hypothesis predicts that tumors are constituted of a highly heterogeneous population of genetically

distinct cell variants within which malignant cells harboring drug resistant mutations are likely to pre-exist[336]. Consequently, it has been assumed that MMR deficient tumors are prone to develop secondary resistance to any oncogene-targeted therapy although this statement based on theoretical assumptions still lack experimental and clinical evidences.

General conclusion

Defective MMR plays a critical role in the initiation, progression and therapeutic response of cancers from diverse origins (colorectal, endometrial, brain and hematological) (Figure 10). At early tumorigenesis, defective apoptosis in response to endogenous DNA damage-induced stress endows MMR-deficient cells with a slight proliferative advantage and contributes to their expansion within the malignant population. In more advanced stages, adaptation to selective environmental pressures related to tumor growth as well as immunity evasion are favored by the elevated random mutagenesis readily detectable as MSI. MMR-deficient cancer cells are armored against anti-cancer treatments. On the one hand, as they tolerate DNA damages, genotoxic chemotherapeutics will face primary refractoriness. On the other hand, progressive enrichment of MMR-deficient cells with increasing genetic instability results in a growing number of genetically distinct tumor subclones, which theoretically raises the probability of pre-existing variants harboring drug-resistant mutations that will lead to rapid relapse after oncogene-targeted therapy.

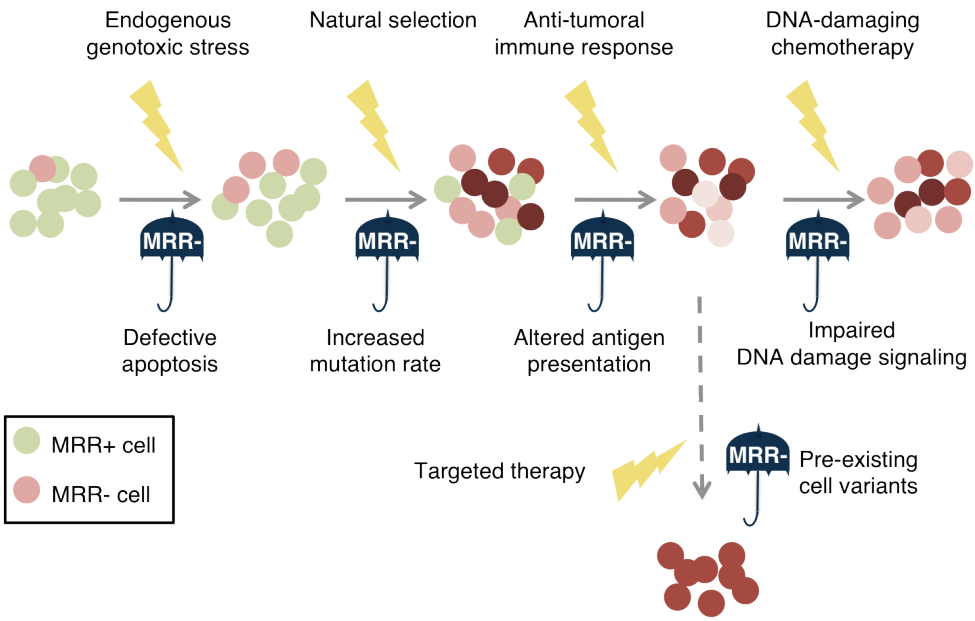


Figure 10. MMR-deficiency plays an active role in tumorigenesis. At the onset of cancer, DNA damages induced by endogneous genotoxic stress fail to elicit apoptosis in MMR-deficient, which results in their slight expansion. As the tumor progresses, malignant cells are exposed to environmental constraints that can be partially counteracted by MMR-deficiency, leading to a progressive enrichment in MMR-deficient cells within the tumor population. MMR-deficiency also influences the response to anti-cancer therapies.

OBJECTIVES

OBJECTIVES

Previous discoveries reported by my host laboratory demonstrated that IL-9 exerted no proliferative activity on freshly isolated lymphocytes but could act as a growth factor for *in vitro* derived T lymphocyte cell lines and promoted their malignant transformation associated with constitutive activation of the JAK-STAT pathway[337]. TS1 is one of such IL-9-dependent cell lines derived from a murine CD4+ T helper clone, which initially required antigen presentation by feeder cells for *in vitro* growth. However, upon long term culture, cells gradually lost their need for antigen stimulation and acquired compensatory IL-9 responsiveness allowing the derivation of stable IL-9-dependent cell lines[338]. The multistep deregulation process undergone by T cells during adaptation to IL-9 was accompanied by a de-differentiation state with the progressive disappearance of T-cell markers reminiscent of pre-transformed cells[339]. Indeed, the IL-9-responsive TS1 cell line spontaneously gave rise to growth factor-independent clones with *in vivo* tumorigenic potency[340] showing constitutive phosphorylation of JAK1, JAK3, STAT3 and STAT5 comparable to those elicited by IL-9 stimulation in parental cells[341]. When I started my PhD project, the causative mechanism remained to be elucidated.

In a first study, we sought to unravel the genetic alterations underlying constitutive JAK-STAT signaling in our collection of growth factor-independent TS1 clones and aimed at evaluating the efficacy of short-term and long-term treatment with JAK inhibitors. This work highlighted the particular genotypic plasticity of TS1 cells endowing them with a remarkable faculty of adaptation to environmental selective pressures (cytokine withdrawal and JAK inhibitors) by successively acquiring activating point mutations in JAK1 and JAK3. As mentioned in the introduction, the emergence of tumor cells harboring newly acquired mutations accounts for the majority of clinical relapses upon TKI therapies. This led us, in a second study, to investigate the molecular mechanism promoting spontaneous mutagenesis in the TS1 model with the hope to find clinically relevant observations.

RESULTS

RESULTS

1. Cooperating JAK1 and JAK3 mutants increase resistance to JAK inhibitors

Cooperating JAK1 and JAK3 mutants increase resistance to JAK inhibitors

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Key Points

- Cells transformed by activating JAK1 mutations become resistant to JAK inhibitor by acquiring activating mutations in JAK3 and vice versa.
- JAK1 and JAK3 mutants cooperatively activate STAT transcription factors.

The acquisition of growth signal self-sufficiency is 1 of the hallmarks of cancer. We previously reported that the murine interleukin-9–dependent TS1 cell line gives rise to growth factor–independent clones with constitutive activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Here, we show that this transforming event results from activating mutations either in JAK1, JAK3, or in both kinases. Transient and stable expression of JAK1 and/or JAK3 mutants showed that each mutant induces STAT activation and that their coexpression further increases this activation. The proliferation of growth factor–independent TS1 clones can be efficiently blocked by JAK inhibitors such as ruxolitinib or CMP6 in short-term assays. However, resistant clones occur upon long-term culture in the presence of inhibitors. Surprisingly, resistance to CMP6 was not caused by the acquisition of secondary mutations in the adenosine triphosphate–binding pocket of the JAK mutant. Indeed, cells that originally showed a JAK1-activating mutation became resistant to inhibitors by acquiring another activating mutation in JAK3, whereas cells that originally showed a JAK3-activating mutation became resistant to inhibitors by acquiring another activating mutation in JAK1. These observations underline the cooperation between JAK1 and JAK3 mutants in T-cell transformation and represent a new mechanism of acquisition of resistance against JAK inhibitors. (*Blood.* 2014;124(26):3924-3931)

Introduction

Hematopoiesis is a tightly regulated process in which cytokines dictate fate (apoptosis, proliferation, or differentiation) of the various progenitors through binding to specific receptors at the surface of target cells. Most hematopoietic cytokine receptor chains associate with a member of the Janus kinase (JAK) family to form a competent cytokine–receptor complex. The JAK family comprises 4 tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) that bind to a proper repertory of preassociated homo- or heterodimeric receptor chains. Structurally, JAK kinases are characterized by the presence of a pseudo-kinase domain directly adjacent to the kinase domain and are supposed to prevent inappropriate catalytic activity of the kinase domain in the absence of stimulus.[1] The major substrates of activated JAKs are the cytoplasmic signal transducer and activator of transcription (STAT) molecules.

STAT factors play an active role not only in physiological hematopoiesis, but also in tumoral transformation because uncontrolled STAT activation leads to growth signal self-sufficiency.[2] In hematological malignancies, a common mechanism for constitutive STAT activation is the deregulation of tyrosine kinase activity of the cytokine–receptor complex by gain-of-function alterations affecting the JAK itself or its associated receptor chain. A classical illustration is the activating $JAK2^{V617F}$ mutation identified in a substantial proportion of patients with chronic myeloproliferative neoplasms (MPNs).[3-6] Among the $JAK2^{V617F}$-negative MPNs, 5% of essential thrombocythemia patients harbor a mutation in the thrombopoietin receptor (commonly MPL^{W515L} or ^{W515K}).[7] In acute leukemias from lymphoid or myeloid origin, various JAK1, JAK2, and JAK3 mutations and translocations have been reported.[8-18] In childhood acute lymphoblastic leukemia (ALL), gain-of-function mutations of the interleukin-7 receptor chain (IL-7Rα) were described in 10% of cases.[19,20] JAK inhibitors therefore represent an appealing therapy for leukemia patients because a significant proportion (20% to 30% in childhood ALL) seems to acquire growth signal self-sufficiency through constitutive activation of the JAK-STAT pathway. Nevertheless, their efficacy has first to be experimentally assessed using preclinical leukemia models.

TS1 is an IL-9–dependent cell line derived from a murine $CD4^+$ T-helper clone. Although normal T cells require antigen presentation for in vitro growth, repeated biweekly antigen stimulation led to gradual acquisition of IL-9 responsiveness, loss of the need for antigen presentation, and emergence of permanent IL-9–dependent cell lines such as TS1.[21] The progressive deregulation undergone

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by T cells during acquisition of IL-9 responsiveness is somewhat reminiscent of the multistep process leading to cell transformation.[22,23] Furthermore, the IL-9–responsive TS1 cell line could spontaneously give rise to growth factor (GF)-independent clones that were tumorigenic in vivo.[24] Even though there was no autocrine cytokine production in the supernatants of these GF-independent clones, they showed constitutive phosphorylation of JAK1, JAK3, STAT3, and STAT5 comparable to those induced by IL-9 stimulation in parental TS1 cells.[25]

Because of its T-cell origin and its ability to spontaneously recapitulate the transformation process in vitro, the TS1 model provides us with a unique opportunity to identify T-cell–specific oncogenic events potentially implicated in ALL. The aims of our study were first to unravel the genetic mechanisms underlying constitutive JAK-STAT activation in this model and, second, to test the efficacy of short- and long-term treatment with JAK inhibitors.

Methods

Cell cultures

TS1 and BW5147 cells were cultured in Iscove-Dulbecco's medium supplemented with 10% fetal bovine serum, 50 μM β-mercaptoethanol, 0.55 mM L-arginine, 0.24 mM L-asparagine, 1.25 mM L-glutamine, and, for TS1 cells, murine IL-9 (100 U/mL) was added. CMK cells were cultured in RPMI medium supplemented with 10% fetal bovine serum.

Stable DNA transfections and selection of clones

Wild-type (WT) JAK3 and human IL-9Rα complementary DNA (cDNA) were subcloned into the pMX-IRES-CD4 biscistronic retroviral vector upstream of the IRES. WT JAK1 and human γ chain (γc) cDNA were subcloned into the pMX-IRES-GFP biscistronic retroviral vector upstream of the IRES. The JAK3 (R657H, V674A) and JAK1 (Y652H and A634D) mutant cDNA were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene). For stable transduction, retroviral supernatants were generated by transient transfection of the BOSC packaging cell line and used for infection of 0.5×10^6 cells as described.[26] Populations of cells expressing markers were isolated by FACS sorting, using a phycoerythrin-coupled antibody against hCD4 (#555347, BD Pharmingen) diluted 1/30.

For the selection of GF-independent TS1 clones, IL-9–dependent TS1 cells were washed 3 times in phosphate-buffered saline and seeded in 96-well plates at a concentration of 5000 cells/well in the absence of cytokines. After 1 to 2 weeks, GF-independent clones were picked up from plates in which less than 20% of wells were positive for proliferation, corresponding to a probability of clonality superior to 0.9 according to the Poisson distribution. For the selection of JAK inhibitor–resistant subclones, GF-independent TS1 clones were seeded in 96-well plates at a density of 30 000 cells/well in the presence of 300 to 600 nM of either pan-JAK inhibitor CMP6 (Calbiochem, cat#420097) or ruxolitinib (Haoyuan Chemexpress Co., INCB018424). After 2 to 3 weeks, wells containing proliferating cells were counted. Resistant cells from plates meeting clonality conditions were picked up and amplified in the presence of the respective JAK inhibitor.

Luciferase assays

STAT5 transcriptional activity was assessed by measurements of luciferase expression in HEK293 cells upon transient transfection of appropriate cDNA and pLHRE-luc vector harboring tandem copies of the STAT5-inducible lactogenic hormone response element of the rat β-casein gene promoter, inserted upstream a luciferase gene.[27] Another reporter plasmid, Renilla luciferase (pRLTk, Promega), was cotransfected as an internal transfection control. Transient transfection of HEK293 cells by LipofectAMINE (Invitrogen) was previously described.[28] Twenty-four hours after transfection, luciferase assays were performed using the Dual Luciferase Reporter Assay kit (Promega).

Western blots

For western blot analysis, 2×10^6 BW5147 or CMK cells were lysed in 200 μL of lysis buffer containing 50 mM tris(hydroxymethyl)aminomethane-HCl, 1% NP40, 150 mM NaCl, 1 mM EDTA, 15% glycerol, Halt Protease Inhibitor Cocktail 1/100, phenylmethylsulfonyl fluoride 0.1 mM, and sodium orthovanadate 0.1 mM. A total of 35 μL of lysates was loaded on Bolt 4% to 12% Bis-tris precast gels (Novex) and electrophoretically transferred to nitrocellulose membranes (i-blot Gel Transfer Stacks, Novex). Phosphorylation of signaling proteins was investigated with the following phospho-specific antibodies from Cell Signaling Technology: anti-pY705 STAT3 (#9131) and anti-pY694 STAT5 (#9351). Blots were reprobed with anti-JAK1 (#3332), anti-JAK3 (#3775), anti-STAT3 (#9132) (Cell Signaling Technology), anti-STAT5 (#835, Santa Cruz), or anti-β-actin (#A5441, Sigma) antibodies as a control.

RNA extraction, cDNA synthesis, PCR, and sequencing

Total RNA was isolated from 10^6 TS1 cells using TriPure Isolation Reagent (Roche). Reverse transcription was performed on 1 μg of total RNA using an oligo-dT primer and M-MULV Reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Scientific). Taq DNA polymerase-mediated polymerase chain reaction (PCR) amplification (TaKaRa) was performed and PCR products were sequenced using the DYEnamic Big Dye Terminator Kit (Amersham).

Results

GF-independent TS1 clones acquired point mutations in the pseudo-kinase and kinase domains of JAK1 and/or JAK3

TS1, a T-lymphocyte cell line, is dependent on IL-9 for proliferation. Removal of IL-9 from the culture medium results in rapid cell death, but few cells occasionally succeed to proliferate independently of GF (at a frequency of approximately 1/20 000). We selected 50 GF-independent TS1 clones. Constitutive STAT5, STAT3, AKT, JAK1, and JAK3 phosphorylation could be observed in all tested IL-9–independent clones, but not in IL-9–starved parental TS1 cells[25] (supplemental Figure 1, available on the *Blood* Web site). GF-independent TS1 clones remained sensitive to JAK inhibitors, indicating that their proliferation still relies on JAK activity (data not shown).

Because both JAK1 and JAK3 were constitutively phosphorylated, we sequenced the JAK1 and JAK3 cDNA in our collection of 50 GF-independent TS1 clones and found that all of them had acquired a missense point mutation affecting a residue of the pseudo-kinase or kinase domains of either JAK1, JAK3, or both (Table 1). A majority of clones harbored a single mutation in 1 JAK (23 with JAK1 mutation vs 22 with JAK3 mutation), but some clones (5 of 50) acquired a mutation in both kinases of the IL-9 receptor complex. Altogether, we identified 16 different mutations (6 in JAK1 and 10 in JAK3) affecting 14 residues conserved in mouse and human JAK. As shown in Figure 1, the great majority of mutations map to the interface between pseudo-kinase and kinase domains of JAK1 and JAK3 that was recently shown to mediate autoinhibitory mechanisms.[29] For 7 of the 14 residues mutated in our clones, mutations have been also described in human cancers (supplemental Table 1). Although the total number of mutations of JAK1 and JAK3 was equal (28 JAK1 mutations vs 27 JAK3 mutations), statistics confirm that they were not randomly distributed among experiments suggesting that the relative expansion of the mutated cells occurred before

Table 1. List of the JAK1 and JAK3 mutations identified in GF-independent TS1 clones

JAK1	JAK3	Number of TS1 clones (n = 50)
V645A*	—	1
Y652H*	—	16
Y652D*	—	1
K847E	—	4
F958V	—	1
—	A572V*	1
—	L586S	6
—	R657H*	2
—	F666L	1
—	V674A*	10
—	Y824C*	1
—	T848A	1
S646P*	L586V	1
Y652H*	R657H*	1
Y652H*	Y904C	1
Y652H*	Y939C	1
F958V	Y824C*	1

The 16 different mutations occurring either separately or associated. For each combination, the absolute frequency is indicated for 50 characterized TS1 clones. All 14 affected amino acids are conserved in mouse and human JAK (amino acid numbers correspond to human sequence).

*Described in human cancers.

cytokine withdrawal (chi-squared test, $P < .05$) (supplemental Table 2).

Activated JAK1 and JAK3 mutants cooperate for downstream signal transduction

The observation that some GF-independent clones had acquired mutations both in JAK1 and JAK3 raised the hypothesis that these mutants might cooperatively contribute to the activation of the signaling pathways necessary for survival and proliferation. To address this hypothesis, we compared the activation of the JAK-STAT pathway induced by expression of a single JAK mutant vs coexpression of the JAK1^{Y652H} together with JAK3^{V674A} or JAK3^{R657H} mutants found in ALL patients. To avoid any bias during selection of stable transfectants, these mutants were expressed in the murine lymphoma T cells BW5147, which do not rely on the JAK-STAT pathway for proliferation.

As shown in Figure 2A, stable expression of JAK1^{Y652H}, JAK3^{V674A}, or JAK3^{R657H} alone resulted in constitutive phosphorylation of STAT5 (Figure 2A, lanes 3, 4, and 6). This phosphorylation was further increased when JAK1 and JAK3 mutants were coexpressed (Figure 2A, lanes 5 and 7). For JAK1, JAK3, and STAT3, phosphorylation was either exclusively observed in the presence of a combination of 2 mutants or increased in comparison with each mutant separately. This indicates that, in BW5147 cells, JAK1 and JAK3 mutants cooperate in their transphosphorylation and subsequent activation of downstream signaling molecules STAT3 and STAT5. These observations were corroborated in the human acute megakaryoblastic leukemia CMK cell line harboring a heterozygous activating mutation in JAK3 (A572V).[13] As displayed in Figure 2B, stable expression of JAK1^{A634D} enhanced the phosphorylation of JAK3 and STAT3 providing evidence that a JAK1 activated mutant can collaborate with an endogenous JAK3 mutant.

Furthermore, in HEK293 cells transiently transfected with a STAT5-responsive luciferase reporter construct, single expression of JAK1^{Y652H}, JAK3^{V674A}, or JAK3^{R657H} induced STAT5 activation that was further exacerbated by coexpression of JAK1 and JAK3 mutants (Figure 3). Altogether, these 2 different experimental models show the cooperation of JAK1 and JAK3 mutants in the activation of the JAK-STAT pathway in 3 cell lines and support the hypothesis that JAK1 and JAK3 collaborate toward the transformation of IL-9–dependent TS1 cells into GF-independent clones.

Secondary mutations in the other JAK of the cytokine receptor complex confer increased resistance to JAK inhibitors

CMP6 is an adenosine triphosphate (ATP)-competitive tyrosine kinase inhibitor that effectively inhibits all JAK. In short-term assay, GF-independent proliferation of TS1 clones could be efficiently blocked by CMP6 with a half maximal inhibitory concentration (IC_{50}) close to 0.1 μM (data not shown). GF-independent TS1 clones harboring primary mutations in either JAK1 (A3, A4) or JAK3 (A6, A11, T4, T15) were seeded in 96-well plates in the presence of CMP6 concentrations inhibiting 100% of cell proliferation within 2 days (300 to 600 nM). Upon long-term culture, CMP6-resistant clones arose at a low frequency (1 in 2 million cells). Using this experimental setup, 20 CMP6-selected subclones were obtained from 2 independent TS1 clones with the JAK1^{Y652H} mutation, and 56 CMP6-selected subclones were obtained from 4 independent clones with 3 different JAK3 mutations (L586S, V674A, and T848A). Contrary to

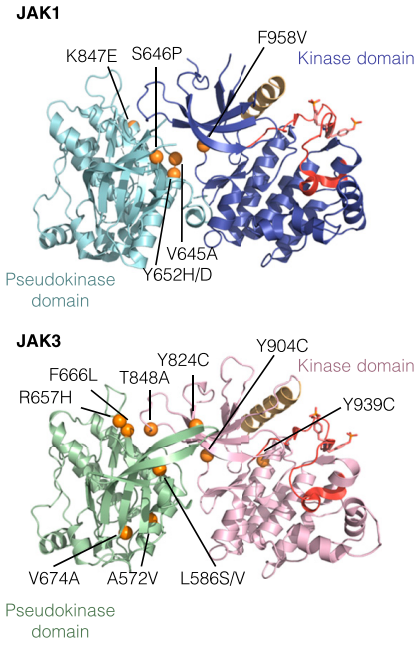


Figure 1. Localization of the 16 identified missense point mutations in JAK1 and JAK3. The figure represents the predicted 3-dimensional structure of kinase and pseudo-kinase domains of JAK1 and JAK3 based on the recent TYK2 crystal structure.[29] Molecular images were obtained with the PyMOL molecular visualization system. The pseudo-kinase domain is shown in light blue for JAK1 and green for JAK3. The adjacent kinase domain is shown in violet for JAK1 and pink for JAK3. The αC helix is shown in beige and activation loop in red. Mutated residues are indicated with orange balls.

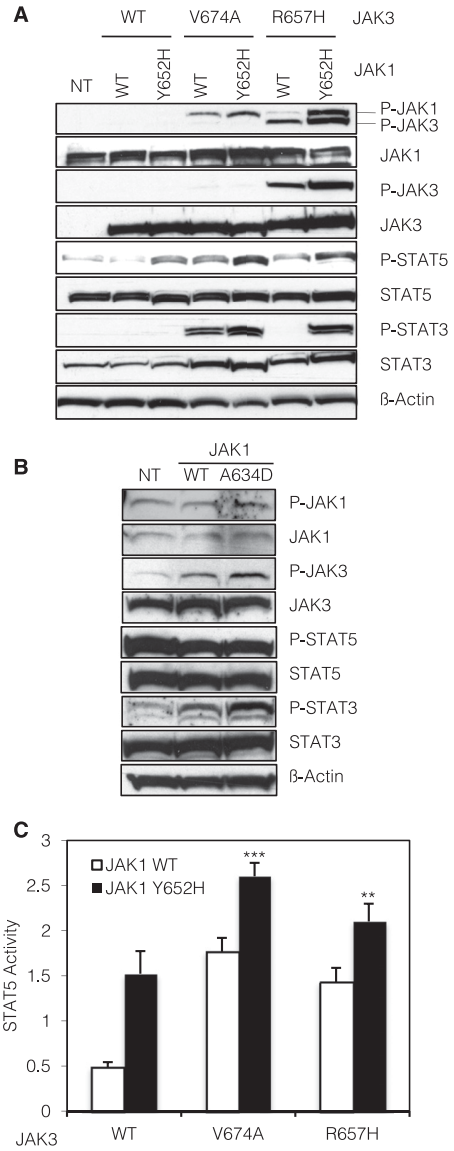


Figure 2. JAK1 and JAK3 mutants cooperatively activate the JAK-STAT pathway. (A) A total of 2×10^6 BW5147 cells nontransduced (NT) or stably transduced with JAK1 (WT or Y652H mutant) together with JAK3 (WT, V674A, or R657H mutants) were lysed and subjected to western blot analysis. The basal phosphorylation level of STAT and JAK proteins was detected using anti-pY694 STAT5, anti-pY705 STAT3, anti-pY1034/1035 JAK1, and anti-pY980/981 JAK3 antibodies. Cells expressed equivalent levels of JAK1 and JAK3, as shown with anti-JAK1 and anti-JAK3 antibodies. Membranes were reprobed with anti-STAT3, anti-STAT5, and anti–β-actin antibodies as loading control. (B) A total of 2×10^6 CMK cells NT or stably transduced with JAK1 (WT or A634D mutant) were lysed and subjected to western blot analysis. Basal phosphorylation level of STAT and JAK proteins was detected using anti-pY694 STAT5, anti-pY705 STAT3, anti-pY1034/1035 JAK1, and anti-pY980/981 JAK3 antibodies. Membranes were reprobed with anti-STAT3, anti-STAT5, anti-JAK1, anti-JAK3, and anti–β-actin antibodies as loading control. (C) HEK293 cells were transiently cotransfected with JAK1 (WT or Y652H mutant), JAK3 (WT, V674A, or R657H mutants), the IL-9Rα chain, and common γ-chain, in addition to the STAT5-responsive luciferase reporter construct pLHRE and the pRLTK plasmid as transfection control. Twenty-four hours posttranfection, cells were lysed and subjected to dual luciferase activity assay. Histograms are means ± standard error of the mean of 5 independent experiments. A 1-way analysis of variance test was performed to compare the condition with the 2 mutants vs each condition with 1 mutant only (**$P < .01$).

our expectations, none of these subclones had acquired any secondary mutations in the originally mutated JAK. By contrast, 16 of the 20 subclones (80%) with a primary JAK1 mutation had acquired a mutation in JAK3, whereas 32 of the 56 subclones (57%) with a primary JAK3 mutation had acquired a mutation in JAK1 (Table 2).

Most of the secondary mutations affect the homologous residues F958 in JAK1 and Y904 in JAK3 located in the ATP-binding pocket of the kinase domain. We previously showed that mutations targeting the F958 residue of JAK1 not only confer constitutive kinase activity but also resistance to ATP-competitive JAK inhibitors.[30] However, other secondary mutations were located in the pseudo-kinase domain of JAK1 (Y652H and F804L) or in the kinase domain of JAK3 outside the ATP-binding pocket (R840C) and are not expected to directly confer resistance to JAK inhibitor. For instance, the $JAK1^{Y652H}$ mutation was previously shown to transform BaF3 cells but did not change their sensitivity to JAK inhibitors.[30] As illustrated in Figure 3A-B, the proliferation of TS1 clones harboring the primary $JAK1^{Y652H}$ mutation (clones A3 and A4) was blocked by CMP6 with an $IC_{50} \leq 0.1$ μM in the same range as clones with the $JAK3^{V674A}$ or $JAK3^{T848A}$ mutations (clones A11 and T15, respectively). However, the CMP6-selected subclones A11R21 and T15R3, which both had acquired the $JAK1^{Y652H}$ as a secondary mutation, showed a 4 to 7 times higher IC_{50} of 0.4 to 0.7 μM. This observation indicated that increased resistance to a kinase inhibitor such as CMP6 can be conferred by cooperativity between 2 active mutants of JAK1 and JAK3, which are both intrinsically CMP6-sensitive.

To test this hypothesis in another model, we took again advantage of the human CMK cell line carrying the $JAK3^{A572V}$ mutation. Proliferation of CMK cells can be efficiently blocked by the JAK1/2 inhibitor ruxolitinib. However, CMK cells transduced with the $JAK1^{A634D}$ mutant exhibited a 2-fold shift in IC_{50} compared with cells transduced with JAK1 WT or nontransduced (nontransduced: 88 nM; JAK1 WT: 75 nM; $JAK1^{A634D}$: 163 nM) (data not shown).

These experiments show that acquiring a secondary activating mutation in another JAK allows for increased resistance to JAK inhibitors that block both JAKs. However, a striking observation from the CMP6-selected subclones described in Table 2, is that such secondary mutations did not occur in the other allele of the originally mutated JAK. This contrasts with $JAK2^{V617F}$-MPNs patients, where homozygosity is often observed because of homologous recombination. JAK2 associates with homodimeric receptors such as the erythropoietin-receptor, whereas JAK1 and JAK3 are involved in signaling by heterodimeric cytokine receptor complexes using the common γc. In this respect, our data suggest that signaling by heterodimeric receptor complexes is further boosted by acquiring another heterozygous activating mutation in the JAK partner rather than by having both alleles of a single JAK mutated.

To address this question, we reconstituted homozygous and heterozygous JAK configurations in HEK293 cells by transiently

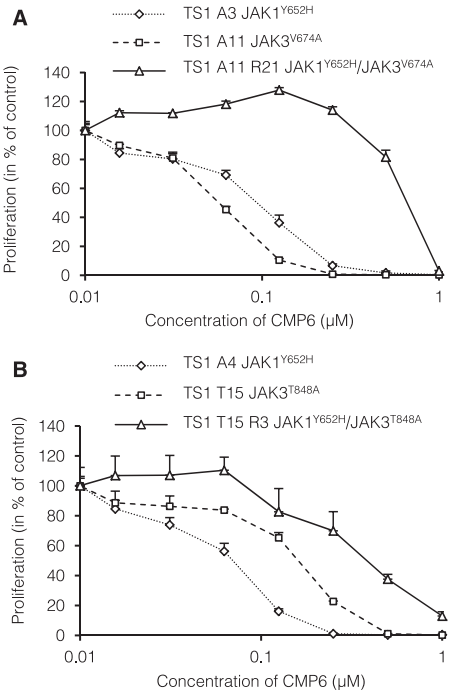


Figure 3. Sensitivity to pan-JAK inhibitor CMP6 of GF-independent and CMP6-selected TS1 clones. (A-B) GF-independent TS1 clones with endogenous JAK1 or JAK3 mutants and CMP6-selected TS1 clones with endogenous JAK1 and JAK3 mutants were seeded in 96-well plates at a density of 1000 cells/well with increasing concentrations of pan-JAK inhibitor CMP6 (0 to 1 μM). After 48 hours, methyl-^{3}H thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results are means ± standard deviation of triplicate cultures represented in % of control untreated cells.

transfecting JAK1 (WT, Y652H, or an equimolar mix of both) and JAK3 (WT, V674A, or both) together with the IL-9Rα and γc cDNA. Cells were then treated or not with increasing concentrations of CMP6 (0.01 to 0.1 μM) and STAT5 activity was assessed 24 hours posttransfection. As shown in Figure 4, the basal level of STAT5 activity was higher in cells having 50% from each activated JAK mutant compared with cells having 100% of mutated JAK1 or JAK3 (black histograms). This boost in JAK-STAT pathway activation could be explained by the fact that both JAK1 and JAK3 mutants interact within the same heterodimeric receptor complex, whereas such an association is not possible in a homozygous situation. CMP6 treatment decreased STAT5 activity in all transfected cells but for each inhibitor concentration, STAT5 activity remained significantly higher in the double heterozygous condition compared with the homozygous ones (Figure 4). Complete titration allowing to assess IC_{50} concentrations confirmed that the intrinsic ability of CMP6 to inhibit STAT5 phosphorylation is not altered in the double heterozygous condition (JAK1 homozygous: 61 nM; JAK3 homozygous: 365 nM; double heterozygous: 250 nM) (data not shown). These results support the hypothesis that acquiring a secondary mutation in the JAK partner from heterodimeric cytokine receptor complex activates the pathway further than becoming homozygous. In addition, when basal STAT5 activity is boosted to strong levels by the 2 mutated JAKs, CMP6 still interferes with STAT5 activation, but higher concentrations would be required to reach a critical threshold.

Secondary mutations in JAK3 confer increased resistance to a JAK1-specific inhibitor

It is well known that the transforming effect of an active JAK mutant depends on its association with cytokine receptor chains allowing homo- or heterodimerization with another JAK and subsequent cross-phosphorylation.[31,32] For instance, an active JAK3 mutant requires a functional JAK1 partner for constitutive signaling.[33] When CMP6, a pan-JAK inhibitor, was used to select resistant TS1 subclones, selective pressure was exerted on both the mutated JAK and its partner, and secondary mutations in the JAK partner provided a selective advantage. We thus wondered whether the same process of selection would occur if only JAK1 activity is blocked with a more selective kinase inhibitor such as ruxolitinib, which is at least 100 times less potent on JAK3.[34]

To address this question, we seeded 6 different GF-independent TS1 clones harboring the primary JAK1^{Y652H} mutation in the presence of ruxolitinib. Ruxolitinib-resistant subclones arose at a frequency comparable to what was observed during selection with CMP6. JAK1 and JAK3 cDNA sequencing revealed that 44 of 53 ruxolitinib-selected TS1 subclones had acquired secondary mutations in the JAKs. Twenty-five of them (57%) had a secondary mutation in JAK3, similar to the selection with CMP6, although ruxolitinib is not supposed to block JAK3. However, contrasting with CMP6 selection, 18 of the ruxolitinib-selected subclones had a secondary mutation in JAK1, either within or outside the ATP-binding pocket, including 4 subclones that became homozygous for the JAK1^{Y652H} mutation (Table 3). Finally, 1 of the ruxolitinib-selected subclones had acquired 2 secondary mutations, JAK1^{F958S} and JAK3^{R840C}. As for the primary mutations, the

Table 2. List of secondary mutations occurring in CMP6-selected TS1 subclones derived from 6 different GF-independent TS1 clones

	Primary mutation		Secondary mutation		
TS1 clones	JAK1	JAK3	JAK1	JAK3	Frequency
A3	Y652H	—	—	Y904H	7/10
			—	Y904C	1/10
			—	—	2/10
A4	Y652H	—	—	R840C	1/10
			—	Y904H	1/10
			—	Y904C	6/10
			—	—	2/10
A6	—	L586S	F958C	—	3/6
			F958V	—	3/6
A11	—	V674A	Y652H	—	1/27
			F958C	—	4/27
			F958V	—	1/27
			F958L	—	1/27
			—	—	20/27
T4	—	V674A	F958C	—	10/13
			F958V	—	3/13
T15	—	T848A	Y652H	—	4/10
			F804L	—	1/10
			F958C	—	1/10
			—	—	4/10

The autonomous TS1 clones with their corresponding primary JAK mutation and the secondary mutations identified in their CMP6-selected subclones. For each combination, relative frequency is indicated.

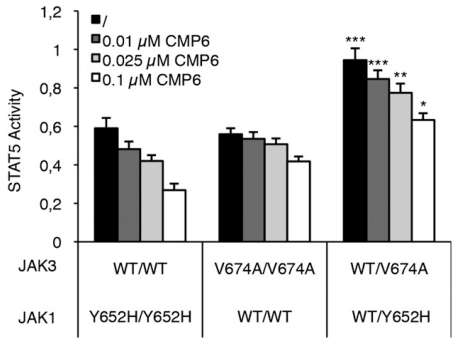


Figure 4. Inhibition by CMP6 of STAT5 activity in cells expressing JAK1 and/or JAK3 mutants. HEK293 cells were transiently cotransfected with JAK1 (WT, Y652H mutant, or both in equimolar concentration to mimic heterozygous vs homozygous configuration), JAK3 (WT, V674A mutant, or both), IL-9Rα, and γc, in addition to the STAT5-responsive luciferase reporter construct pLHRE and the pRLTK plasmid as transfection control. Cells were maintained in the presence of increasing concentrations of pan-JAK inhibitor CMP6 or left untreated. Twenty-four hours posttransfection, cells were lysed and subjected to dual luciferase activity assay. Raw values were standardized with BCR-ABL–induced STAT5 activity. Histograms are means ± standard error of the mean of 5 independent experiments performed in triplicate. A 1-way analysis of variance test was used to determine *P* values between the heterozygous and the 2 homozygous configurations for each CMP6 concentration (*$P < .05$, **$P < .01$, ***$P < .001$).

observation of a single predominant mutation within an experiment (eg, JAK3^{Y824D} in A3) is consistent with the hypothesis of preexisting secondary mutations before selective pressure with the inhibitor. Altogether, our results indicate that when 1 JAK from a heterodimeric cytokine receptor complex is specifically targeted by a tyrosine kinase inhibitor, resistance can be conferred either by a secondary mutation in the other JAK partner or by a secondary activating mutation in the same JAK. In both cases, and in line with CMP6-selected clones, the secondary mutations are not restricted to the ATP-binding pocket.

Discussion

In the present study, we describe a new mechanism of resistance to JAK kinase inhibitors. We show that 2 concomitant spontaneous activating mutations in endogenous JAK1 and JAK3 cooperatively activate the JAK-STAT pathway leading to increased resistance to CMP6 or Ruxolitinib. Interestingly, such resistance could be conferred by combining mutations that do not affect the sensitivity of cells to JAK inhibitors when present individually.

In our series of subclones selected in the presence of tyrosine kinase inhibitors (TKI), TKI resistance was not primarily conferred by impairing drug binding to the oncogenic kinase, but rather by increasing the activity of the heterodimeric JAK–receptor complex. Indeed, secondary mutations associated with CMP6 resistance did not affect the originally mutated JAK. In addition, the secondary mutations in the JAK partner observed here were previously described as activating mutations per se, associated with oncogenesis in vitro and in vivo (supplemental Table 1; Hornakova et al[30]). However, these activating mutations can be further divided into 2 types. First, a series of mutations can either lead to GF independence with normal TKI sensitivity when occurring as primary mutations or increase TKI resistance when occurring as secondary mutations. This first set of mutants is illustrated in Figure 4 with JAK1^{Y652H}. On the other hand, another series of secondary mutations target the JAK1 F958 residue or the JAK3 Y904 residue, which correspond to homologous amino acids that were previously found to confer both constitutive activation and lower sensitivity to JAK inhibitors.[30] Thus, selective pressure with the JAK inhibitor selects essentially for activating mutations, but with a further advantage for gain-of-function mutations that also affect drug binding.

In patients relapsing after treatment with TKI targeting oncogenic proteins such as BCR-ABL, c-Kit and epidermal growth factor receptor (EGFR) secondary mutations frequently occur in the kinase domain of the respective target. A great part of those substitutions affect the conserved threonine gatekeeper residue regulating access of TKIs to the ATP binding pocket, pointing to impaired drug binding as the main cause of resistance. Classic examples include the BCR-ABL^{T315I} mutation in chronic myeloid leukemia (CML),[35] the c-Kit^{T670I} mutation in gastrointestinal stromal tumors[36] and EGFR^{T790M} in lung adenocarcinomas.[37] There are other substitutions described in imatinib-resistant CML patients that are not located near the ATP-binding site of BCR-ABL, but are postulated to destabilize the conformation of the kinase domain required for inhibitor binding.[38]

Interestingly, some of the BCR-ABL imatinib-resistant mutations (Y235F/E255K/T315I) have also been reported to enhance the oncogenic potency or kinase activity of the fusion protein independently of their effect on drug sensitivity and are therefore considered as gain-of-function mutations.[39-41] Using high-sensitivity detection methods, BCR-ABL imatinib-resistant mutations could be detected at low levels in a considerable fraction (12.5% to 22%) of untreated CML patients, indicating that resistant mutations can preexist to the onset of treatment.[42-44] Noticeably, the majority of pretreatment secondary mutations are gain-of-function substitutions,

Table 3. List of secondary mutations occurring in ruxolitinib-selected TS1 subclones derived from 6 different GF-independent clones

	Primary mutation		Secondary mutation		
TS1 clones	JAK1	JAK3	JAK1	JAK3	Frequency
A3	Y652H	—	—	Y824D	8/9
			—	—	1/9
A4	Y652H	—	—	Y904C	2/9
			—	Y904H	3/9
			F958C	—	1/9
			—	—	3/9
A5	Y652H	—	—	L586S	3/11
			—	F666L	1/11
			—	R840H	1/11
			—	Y904C	5/11
			L910P	—	1/11
A7	Y652H	—	Y652H	—	1/10
			F958S	—	8/10
			F958S	R840C	1/10
T16	Y652H	—	—	R840H	1/9
			Y652H	—	1/9
			S1042I	—	2/9
			—	—	5/9
T18	Y652H	—	—	Y904C	1/5
			Y652H	—	2/5
			F958V	—	1/5
			S1042I	—	1/5

The GF-independent TS1 clones with their corresponding primary JAK1 mutation and the secondary mutations identified in their ruxolitinib-selected subclones. For each combination, relative frequency is indicated.

suggesting that they might confer a slight proliferation advantage over parental BCR-ABL in the absence of imatinib, and expand during treatment under selective pressure.[43,44] In our TS1 model, a similar selection process of preexisting activating JAK1 and JAK3 mutations might take place. During culture, spontaneous mutations occur randomly throughout the TS1 cell genome and some of them activate JAK1 or JAK3. GF-independent cells with a single activating JAK mutation that acquired by chance a secondary activating mutation in the JAK partner activate further the JAK-STAT pathway, which provides a subtle growth advantage allowing for their persistence and slow relative expansion in the culture. JAK inhibitor selection pressure will foster the emergence of these double-mutated cells because their higher basal level of JAK-STAT activation will raise the threshold inhibitor concentration needed to induce apoptosis. The hypothesis that the secondary mutations preexisted before selection with JAK inhibitors is supported by the fact that, in several experiments, a majority of TKI-resistant subclones showed the same secondary mutation (Tables 2 and 3).

In ALL patients, activating mutations in either JAK1, JAK3, and IL-7Rα have been described in approximately 20% of the patients,[18,45] raising a potential indication for JAK inhibitors. Our data suggest that such treatment could favor the selection of tumor clones with preexisting mutations in both JAK1 and JAK3. This possibility is supported by the fact that several cases of leukemia patients with concomitant mutations in the pseudo-kinase or kinase domains of JAK1 and JAK3 have already been reported (supplemental Table 3). [18,46-48]

Secondary mutations are not the exclusive way for transformed cells to survive in the presence of TKIs, and some of our TKI-resistant subclones did not show any secondary mutation in JAK1 or JAK3 (Tables 2 and 3). The 2 types of JAK inhibitor–selected subclones (mutated and nonmutated) cannot be distinguished based on their sensitivity to inhibitors. All JAK inhibitor–selected subclones do not exhibit an absolute resistance, but rather a relative shift in sensitivity (Figure 3A-B). This gives us a rationale to speculate that the unknown mechanism allowing the nonmutated subclones to proliferate in the presence of inhibitor still relies on JAK activity. However, no mutations in JAK2 and TYK2 were found in 20 nonmutated subclones analyzed so far. Alternatively, a shift in oncogene addiction from the JAK-STAT toward phosphatidylinositol 3-kinase or mitogen-activated protein kinase pathways is not excluded.

In JAK2^{V617F}-transformed cell lines treated with gradually increasing doses of ruxolitinib in vitro, JAK2 overexpression was associated with a reversible stage of so-called JAK2 inhibitor persistence.[49] In our laboratory, we observed a similar increase in the expression of JAK1 messenger RNA in BAF3 cells expressing the endogenous JAK1^{F958V} mutant when cells were cultured in the presence of CMP6.[30] However, we did not observe any significant increase in JAK1 and JAK3 messenger RNA expression levels for our TKI-resistant subclones without secondary mutations in JAK (data not shown). An alternative resistance mechanism might result from loss of negative regulators of the pathway, such as PTPN2, a phosphatase active on phospho-tyrosine residues located in the activation loop of JAK1 and JAK3 and necessary for full kinase activity.[50] Deletions of PTPN2 were identified in 6% of T-cell ALL cases[51] and knockdown of PTPN2 reduced sensitivity of cells to JAK inhibition.[52]

Because of its high versatility, the TS1 model described here represents an invaluable tool for further studies aimed at unraveling new secondary mutation in such genes that might cause increased TKI resistance.

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Authorship

Contribution: L.S., T.H., E.L., and F.L. carried out experiments, analyzed the data, and performed the statistical analysis; S.N.C., E.F., and M.T. generated and provided mutated JAK constructs; L.S., T.H., S.N.C., M.T., L.K., and J.C.R. conceived the study and participated in the design and coordination of the experiments; E.L. performed the modeling work; L.S., J.C.R., and L.K. wrote the paper; and all authors read and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Supplementary data

Mutation	Tumor type	Proof of Activity
JAK1 V645F	Hepatocellular carcinoma [1]	+ [2]
JAK1 S646F	Childhood ALL [3]	+ [3]
JAK1 Y652H	T-ALL [4]	+ [2]
JAK3 A572V	AMKL [5], NKT-CL [6], T-PLL [7]	+ [5, 6]
JAK3 R657Q	DS-AMKL[8], JMML [9],T-PLL [10]	+ [11]
JAK3 V674A	T-ALL [12],T-PLL [7]	+ [11]
JAK3 Y824D	T-PLL [7]	ND

Supplementary table 1. JAK1 and JAK3 mutants identified in our TS1 model and in human cancers. Proof of activity was defined as the ability to transform BAF3 cells, or to activate STAT factors in HEK293 cells. ALL acute lymphoblastic leukemia, AMKL acute megakaryoblastic leukemia, NKT-CL natural killer T-cell leukemia, T-PLL T-prolymphocytic leukemia, DS-AMKL Down syndrome-related acute megakaryoblastic leukemia, JMML juvenile myelomonocytic leukemia.

	JAK1	JAK3	Mutation occurrence (n=55)
EXP1	Y652H		6/12
	F958V		1/12
		L586S	3/12
		V674A	1/12
		Y935C	1/12
EXP2	V645A		1/16
	S646P		1/16
	Y652H		1/16
	K847E		3/16
		A572V	1/16
		L586V	1/16
		R657H	2/16
		F666L	1/16
		V674A	4/16
		T848A	1/16
EXP3	Y652H		4/9
	F958V		1/9
		R657H	1/9
		Y824C	2/9
		Y904C	1/9
EXP4		L586S	2/6
		V674A	4/6
EXP5	Y652H		3/5
	Y652D		1/5
		V674A	1/5
EXP6	Y652H		6/7
		L586S	1/7

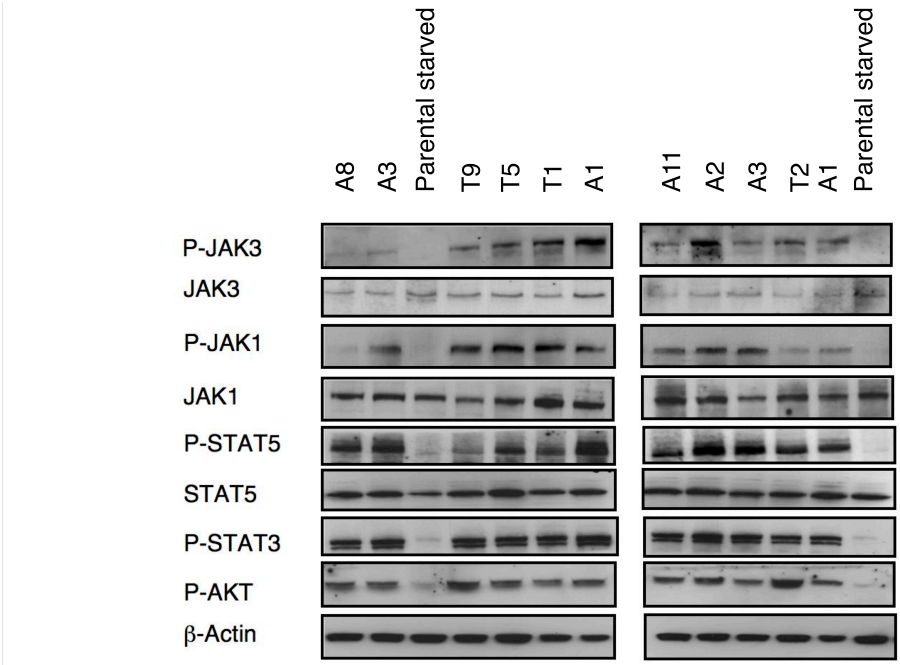
Supplementary table 2. Frequencies of the JAK1 and JAK3 mutations identified in GF-independent clones coming from 6 independent selection experiments.

JAK1	JAK3	Tumor type
Y652D	A572V	NKT-CL[6]
E997K	A572V	T-ALL[12]
L910P	L857P	T-ALL[12]
L653F	M511I	T-PLL[7]
R724H	R657Q	T-ALL[10]

Supplementary table 3. Concomitant JAK1 and JAK3 mutations identified in human leukemia. NKT-CL natural killer T-cell leukemia, T-ALL acute lymphoblastic leukemia, T-PLL T-prolymphocytic leukemia.

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Supplementary figure 1. Growth factor-independent TS1 clones show constitutive activation of JAK1 and JAK3 and downstream signaling molecules. 10^6 cells from starved parental IL-9-dependent TS1 cell line and from 9 GF-independent clones (A1/2/3/8/11 and T1/2/5/9) were lyzed and subjected to Western Blot analysis. Basal phosphorylation level of molecules was detected using anti-JAK3(pY980/981), anti-JAK1(pY1022/1023), anti-STAT5(pY694), anti-STAT3(pY705), anti-Akt(pS473) antibodies. Membranes were reprobed with anti-JAK1, anti-JAK3, anti-STAT5 and anti-ß-Actin antibodies as loading controls.

2. Loss of MLH1 expression promotes the acquisition of oncogenic and inhibitor-resistant point mutations in JAK kinases

Loss of MLH1 expression promotes acquisition of oncogenic and inhibitor-resistant point mutations in tyrosine kinases

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ABSTRACT

Genomic instability drives cancer progression by promoting genetic abnormalities that allow for the multi-step clonal selection of cells with growth advantages. We previously reported that the IL-9-dependent TS1 cell line sequentially acquired activating substitutions in JAK1 and JAK3 upon successive selections for growth-factor independent and JAK inhibitor-resistant cells, suggestive of a defect in mutation avoidance mechanisms. In the first part of this paper, we discovered that the gene encoding MLH1, a key component of the DNA mismatch repair system, is silenced by promoter methylation in TS1 cells. By means of stable ectopic expression and RNA interference methods, we showed that the high frequencies of growth factor-independent and inhibitor-resistant cells with activating JAK mutations can be attributed to the absence of MLH1 expression. In the second part of this paper, we confirm the clinical relevance of our findings by showing that chronic myeloid leukemia relapses upon ABL-targeted therapy correlated with a lower expression of MLH1 messenger RNA. Interestingly, the mutational profile observed in our TS1 model, characterized by a strong predominance of T:A>C:G transitions, was identical to the one described in the literature for primitive cells derived from chronic myeloid leukemia patients. Taken together, our observations demonstrate for the first time a causal relationship between MLH1-deficiency and incidence of oncogenic point mutations in tyrosine kinases driving cell transformation and acquired resistance to kinase-targeted cancer therapies.

INTRODUCTION

Malignant cell transformation requires the acquisition of various aberrant phenotypic hallmarks through the accumulation of advantageous genetic abnormalities, defined as driver mutations[1]. The notion of "mutator phenotype" has been formulated to reconcile the disparity between the low spontaneous mutation rate of normal cells and the thousands of mutations present in a single cancer genome[2]. This concept states that malignant cells exhibit a higher mutation rate that increases the likelihood of acquiring advantageous alterations needed to overcome each selective bottleneck in cancer evolution[2]. As a result, across multiple rounds of selections for driver mutants, there is a simultaneous progressive enrichment of inactivating mutations in caretaker genes that will further enhance random mutagenesis and fuel tumorigenesis.

The mutator hypothesis also has major implications concerning the response to targeted therapies. Indeed, it predicts that cancers

constitute a highly heterogeneous population of stochastically mutated malignant cells within which drug-resistant variants pre-exist and are selected upon treatment causing rapid disease relapse[3]. The importance of such pre-existing mutants is best exemplified in chronic myeloid leukemia patients treated with Imatinib by the emergence of tumors variants with point mutations in the kinase domain of BCR-ABL, which could be detected at low frequency in the bone marrow before treatment[4].

Whether all cancers invariably acquire a mutator phenotype as a driving force for progression is still controversial[5]. However, it is well accepted that certain tumors exhibit a marked genetic instability under the form of chromosomal rearrangements or small deletions/insertions within regions of short tandem repeated nucleotide tracks called microsatellites, a phenotype referred to as microsatellite instability (MSI). For instance, MSI is observed in about 85% of hereditary non-polyposis colon cancers (HNPCC) as a consequence of DNA polymerase slippage errors that are not properly resolved by the mismatch repair (MMR) system[6].

The MMR system guarantees genome fidelity by recognizing and correcting post-replicative errors that escape proofreading activity of DNA polymerases. MMR process is initiated by the binding of a heterodimer composed of MutS homolog-2 and -6 (MSH2 and MSH6) to mispaired or misinserted bases in the newly synthesized DNA strand. Subsequent recruitment of a second heterodimer composed of MutL homolog-1 (MLH1) and post-meiotic segregation protein-2 (PMS2) at the site of the mismatch triggers downstream events that eventually lead to exonuclease-1-mediated removal of the error and resynthesis by DNA polymerases δ or ε[7]. Inefficient MMR confers a strong mutator phenotype characterized by a mutation rate 100- to 1000-fold greater in MMR-deficient cells as compared with normal cells[8-10].

The link between MMR deficiency and tumorigenesis is well established in HNPCC patients who inherit a heterozygous germ-line mutation in one of the MMR components (mainly MSH2 and MLH1) and are prone to cancer development during the fifth or sixth decade of life further to somatic inactivation of the remaining wild-type allele[10]. Rare individuals with inheritance of bi-allelic mutations in the MMR genes present with early childhood onset of hematological and brain malignancies[11]. Besides familial cancers, MMR defects are present in a significant proportion (12-20%) of sporadic counterparts of the HNPCC-spectrum tumors (gastro-intestinal, urologic and gynecologic tracts)[12-15]. More recently, growing evidence for involvement of MMR defects in sporadic hematological malignancies has also been provided[16-22]. In contrast to HNPCC, MMR deficiency in sporadic cancers is commonly caused by silencing of MLH1 owing to promoter methylation.

We previously described a murine lymphoid cell line, called TS1, which was initially dependent on interleukin-9 (IL-9) for proliferation but sequentially gained growth factor (GF)-independence and resistance to JAK-inhibitors at high frequencies (1/20,000 for GF-independent clones and 1 in 2 millions for JAK inhibitor-resistant subclones)[23]. Each of the 2 successive selection steps was accompanied by newly acquired activating point mutations in one of the kinases associated with the IL-9 receptor, namely JAK1 and JAK3. Our data showed that the relative expansion of the mutated cells occurred in culture prior to the selective pressure exerted by cytokine withdrawal or by the inhibitor. The high phenotypic plasticity of the TS1 cell line is somewhat reminiscent of the mutator concept where selection from a large spectrum of randomly pre-existing variants facilitates rapid adaptation to environmental pressures. In the present study, we took advantage of the TS1 model to investigate the molecular mechanisms underlying the acquisition of oncogenic and inhibitor-resistant mutations in JAK kinases.

METHODS

Cell culture

TS1 and TS2 cells were cultured in the presence of murine IL-9 (100U/ml) in Iscove-Dulbecco's medium supplemented with 10% fetal bovine serum, 50μM ß-mercaptoethanol, 0.55mM L-arginine, 0.24mM L-asparagine, 1.25mM L-glutamine. Genome-wide demethylation of TS1 cells was achieved by supplementation of cell medium with 0.5-2μM 5-AZA-2'deoxyCytidine (Sigma-Aldrich, cat#A3656) during 48 hours.

Plasmids construction, cell electroporation and selection of stable transfectants

Murine MLH1 cDNA was inserted into the pEF6V5HisTOPO plasmid (Invitrogen) containing the resistance gene to blasticidin. 10^7cells were electroporated with 50μg of DNA (280V, 1500μF, 75Ω) and selected in the presence of blasticidin 40μg/ml (Invivogen, ANT-BL-1) 24 hours after electroporation.

Retroviral infection with sh-RNA and selection of stable transfectants

Retroviruses were obtained by transient transfection of HEK293 cells with pLKO.1 lentiviral vector coding for shRNA against murine MLH1 (TRCN00000042721, Thermo Scientifics) or anti-GFP (SHC004, Sigma), packaging vector psPAX2 and VSV-G protein vector pMD2-G following the RNAi Consortium recommendations. HEK293 supernatants were harvested at 24 hours and 48 hours post-transfection and used to spin-infect $0.5x10^6$ cells. 3 days after the second infection, stable transfectants were selected with puromycin 5μg/ml (MP biomedicals, cat#194539).

Selection of growth factor-independent clones and JAK inhibitor-resistant subclones

For the selection of GF-independent clones, IL-9-dependent TS1 or TS2 cells were washed 3 times in PBS and seeded in 96-well plates at a concentration of 5,000 cells/well in the absence of cytokines. After 1-2 weeks, GF-independent clones were picked up from plates where less than 20% of wells were positive for proliferation, corresponding to a probability of clonality superior to 0.9 according to the Poisson distribution. For the selection of JAK inhibitor-resistant subclones, GF-independent TS1 clones were seeded in 96-well plates at a density of 30,000 cells/well in the presence of 300-600nM of pan-JAK inhibitor CMP6 (Calbiochem, cat#420097). After 2-3 weeks, proliferation-positive wells from plates meeting clonality conditions were counted.

Microarray

Whole transcripts were amplified from 250ng total RNA and converted into single-stranded DNA using the Ambion® WT expression kit. 5.5μg DNA was fragmented, labeled (Genechip® WT terminal labeling kit, Affymetrix) and hybridized on Genechip® Mouse Genome 1.0 ST array (Affymetrix).

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was isolated from 10^6 cells using TriPure Isolation Reagent (Roche). Reverse transcription was performed on 1μg of total RNA using an oligo-dT primer and M-MULV Reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Scientific). qRT-PCR was performed using the qPCR core-kit for Sybrgreen I (Eurogentec) and primers set annealing to murine MLH1 (5'-AGATGCTCCGTAACCATTCCTTTGTG-3'(fw) and 5'-TAGAACAGCTCTTCACTGAGCTTGGTA-3'(rev)), murine ß-Actin (5′-TCCTGAGCGCAAGTACTCTGT-3′(fw) and 5′-CTGATCCACATCTGCTGGAAG-3′(rev), human MLH1 (5′-ACAGTAGCTGATGTTAGGACACTACC-3′(fw) and 5′-CTATCAGTTCTCGACTAACAGCATTTCC-3′(rev)), human EF1 (5′-GCTTCACTGCTCAGGTGAT-3′ (fw) and 5′-GCCGTGTGGCAATCCAAT-3′ (rev)).

Bisulfite treatment of genomic DNA and sequencing

Genomic DNA was extracted (Dneasy Blood and tissue kit (QIAGEN), digested by EcoRI, and purified with the phenol-chloroform/isopropanol method. Digested DNA was denaturated with NaOH 0.4M (10 minutes at room temperature) before treatment with 3.9M bisulfite solution and 0.5mM hydroquinone during 16 hours (at least 5 cycles of 3 hours at 50°C and 5 minutes at 95°C). The DNA solution was then desalted and concentrated on a silica membrane (GeneClean® DNA purification kit, MP BIOMEDICALS LLC). Alkaline desulfonation was achieved by 0.3M NaOH (10 minutes at room temperature) and neutralized by ammonium acetate. Bisulfite-modified DNA was precipitated with ethanol and Taq DNA polymerase-mediated PCR amplification (TaKaRa) was performed with primers annealing to the modified sequence of the murine promoter of MLH1 (5'-GTTTTTAGAAATGAGTTAATAGGAAGAG-3' (fw) and 5'-TACCAATTTTCTATCATCTCTTTAATAACATTAACC-3' (rev)). PCR products were cloned into the PCR2.1TOPO vector (invitrogen) and transformed into DH5α bacteria. Plasmid DNA was extracted from 7 colonies for each PCR amplification (Nucleospin plasmid-8, Macherey and Nagel) and insert was sequenced using the DYEnamic Big Dye Terminator Kit (Amersham).

Western-blot

For Western blot analysis, $2x10^6$ cells were lysed in 200 µl of lysis buffer containing 50 mM Tris-HCl, 1 % NP40, 150 mM NaCl, 1mM EDTA, 15% glycerol, Halt Protease Inhibitor Cocktail 1/100, PMSF 0.1mM, and Sodium Orthovanadate 0.1mM. 35 µl of lysates were loaded on Bolt 4-12% Bis-Tris pre-cast gels (Novex) and electrophoretically transferred to nitrocellulose membranes (i-blot Gel Transfer Stacks, Novex). Blots were probed with anti-MLH1 (Cell Signaling Technology, #4256) and anti-β-actin (Sigma, #A5441) antibodies.

CML Patient samples

Peripheral blood leucocyte RNA extracts from 8 patients at diagnosis and 8 patients relapsing after one or more TKI were obtained from the Cliniques univeristaires Saint-Luc. Reverse transcription was performed on 1μg of total RNA using an oligo-dT primer and M-MULV Reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Scientific). An independent cohort of 17 diagnostic

and 10 relapse samples obtained from the University of Bordeaux, was used as validation cohort. In all samples, the BCR-ABL/ABL transcript levels were above 10% (IS).

RESULTS

MLH1 expression is repressed by promoter methylation in TS1 cells.

TS1 and TS2 are T-lymphocyte derived cell lines that strictly depend on the presence of IL-9 for survival[24,25] (Figure 1A). IL-9 withdrawal results in rapid cell death in both cell lines though few TS1 cells occasionally succeed to proliferate independently of growth factor at a frequency of 1/20,000 cells. We previously showed that 100% of those GF-independent TS1 clones acquired activating point mutations in JAK1 and/or JAK3[23] suggestive of a high rate of spontaneous mutagenesis. Interestingly, IL-9-starved TS2 cells failed to give rise to GF-independent clones (10^8 cells tested) indicating that this phenotype was specific for the TS1 cell line (Figure 1B).

Based on this observation, we hypothesized that TS1 cells have lost the expression of crucial genome maintenance protein(s). Using a microarray technique, we compared the transcriptomes of TS1 and TS2 cells and obtained a list of 44 genes showing at least a 20-fold differential expression (Figure 1C and supplementary table 1). Among these genes, we identified MLH1 as a relevant candidate because of its pivotal role in MMR, namely coordination of mismatch recognition with effective repair. Expression values for MLH1 transcript were 25-fold lower in TS1 compared with TS2 cells. All other components of the MMR system showed similar expression profiles between TS1 and TS2 cells (data not shown). Loss of MLH1 expression in TS1 versus TS2 cells was confirmed both at the mRNA level by quantitative RT-PCR and at the protein level by western blot analysis (Figures 1D and 1E).

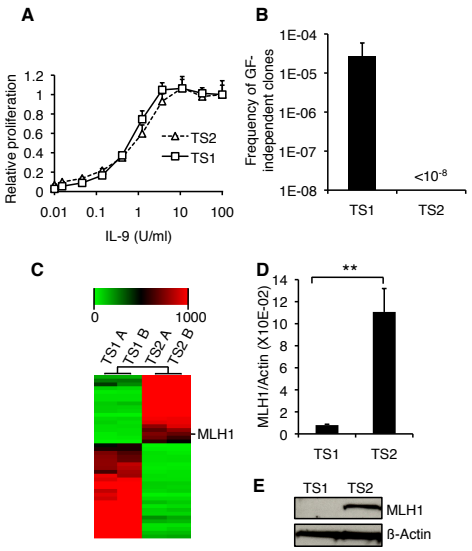


Figure 1. MLH1 expression is lost in TS1 cells. (A) TS1 and TS2 cells were seeded in 96-well plates at a density of 1,000 cells/well in the presence of increasing concentrations of murine IL-9. After 48 hours of culture, methyl-H^3Thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Thymidine incorporation values were standardized relative to the value in the presence of IL-9 100 U/ml. (B) TS1 and TS2 cells were plated in the absence of IL-9 on 96-well plates at different densities ranging from 200 to 40,000 cells/well. 1-2 weeks later, wells positive for proliferation were visually screened. For TS1 cells, histogram represents mean +/- SEM of 7 independent selection experiments. Concerning TS2 cells, no GF-independent clones arose from a total of 10^8 tested cells. (C) Microarray data are represented as hierarchical clustering of transcripts 20X differentially expressed in TS1 versus TS2 corresponding to a list of 44 genes (biological duplicates). The image was obtained with the Multiple experiment Viewer program (MeV). The line corresponding to MLH1 transcript is indicated. (D) MLH1 mRNA levels were assessed by quantitative RT-PCR in TS1 and TS2 cells. Histograms represent means +/- SD of biological triplicates. Student's t-test was performed to determine p-value. (E) MLH1 protein levels were assessed by western blot in TS1 and TS2 cells. The membrane was probed with anti-β-Actin antibody as loading control.

Given that the promoter of MLH1 is frequently hypermethylated in sporadic human tumors, we determined its cytosine methylation status through sequencing of bisulfite-treated genomic DNA. As illustrated in figure 2A, most CpG sites present in the MLH1 promoter region appeared to be methylated in TS1 cells while this promoter was totally unmethylated in TS2. In line with these data, TS1 cells treated with demethylating agent 5-AZA-2'deoxycytidine (5-AZAdC) partially recovered

MLH1 expression in a dose-dependent manner (Figure 2B). Considering those results, we postulated that MMR-deficiency caused by silencing of MLH1 locus accounts for the high rate of spontaneous mutagenesis in TS1 cells.

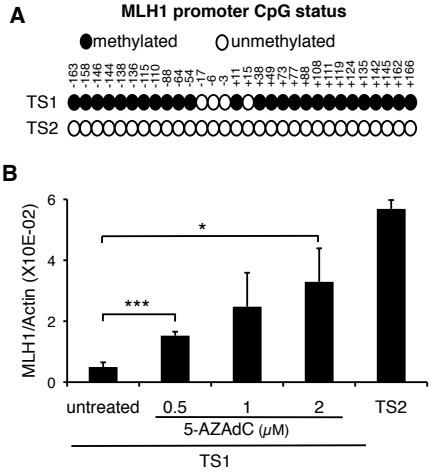


Figure 2. MLH1 expression is repressed by promoter methylation in TS1 cells. (A) Methylation status of the MLH1 promoter was determined by bisulfite sequencing of genomic DNA in TS1 and TS2 cells. Circles represent CpG sites (methylated in black, unmethylated in white). Data represent sequencing results of 7 clones from the PCR product for each cell line. Numbers indicate the nucleotide position relative to the origin of transcription. (B) MLH1 mRNA levels were assessed by quantitative RT-PCR in TS1 cells treated with 0/0.5/1/2 μM of 5-AZA-2'deoxycytidine for 48 hours. Histograms represent means +/- SEM of 3 independent experiments. One-way ANOVA test was performed to determine p-values. (*p<0.05, ***p<0.001)

Ectopic MLH1 expression in TS1 cells prevents oncogenic point mutations in JAK kinases.

In order to demonstrate that loss of MLH1 is responsible for the acquisition of oncogenic mutations, we electroporated IL-9-dependent TS1 cells with an expression vector coding for MLH1 or the empty vector as control. Doing so, we obtained 3 independent sets of MLH1-expressing cells and paired control cells coming from distinct electroporation experiments. Effective restoration of MLH1 was confirmed by western blot on a representative set (Figure 3A). Exogenous MLH1 expression did not modify the proliferation of TS1 cells in response to IL-9 (Figure 3B). By contrast, when MLH1-expressing cells were plated in the absence of IL-9 in order to select GF-independent clones, the frequency of GF-independent clones displays a 50-fold decrease in cells that express MLH1 compared to control cells (Figure 3C). Because in TS1 cells, GF-independence is systematically associated with acquisition of point mutations[23], this difference reflects a reduced mutation rate in MLH1-expressing cells.

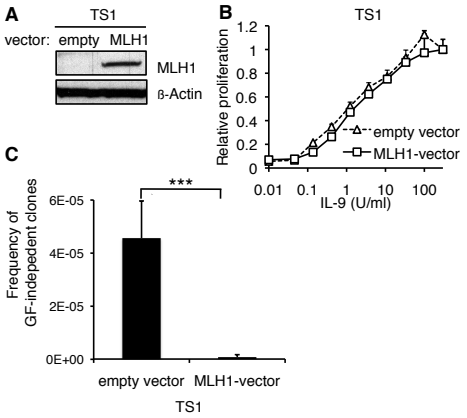


Figure 3. Ectopic MLH1 expression in TS1 cells decreases the frequency of GF-independent clones. (A) MLH1 protein levels were assessed by western blot in TS1 cells electroporated with MLH1 or empty vector. The membrane was probed with anti-β-Actin antibody as loading control. (B) TS1 cells electroporated with MLH1 or empty vector were seeded in 96-well plates at a density of 1,000 cells/well in the presence of increasing concentrations of IL-9. After 48 hours of culture, methyl-H^3Thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Thymidine incorporation values were standardized relative to the value in the presence of IL-9 100 U/ml. (C) TS1 cells were plated in the absence of IL-9 on 96-well plates at different densities ranging from 200 to 20,000 cells/well. 1-2 weeks later, wells positive for proliferation were visually screened. Histograms represent means +/- SEM of 6 independent selection experiments (3 independent electroporated bulks, each bulk was tested twice). Student's t-test was performed to determine the p-value. (***p<0.001)

As previously shown, GF-independent clones were sensitive to JAK inhibitors, such as CMP6, in short-term assays. However, resistant subclones arose upon long-term culture in the presence of inhibitor as a result of secondary mutations in JAK1 or JAK3[23]. Hence, we asked whether restoration of MLH1 in TS1 GF-independent clones could prevent the occurrence of secondary mutations and thereby the emergence of CMP6-resistant subclones. Two different GF-independent clones (TS1 A6 and T4) were electroporated with MLH1-vector (Figure 4A). In short term assays, ectopic MLH1 expression had no impact on the sensitivity of clones to CMP6 (Figures 4B and 4C). However, when cells were seeded in the presence

of the inhibitor for 3 weeks, significantly less CMP6-resistant subclones emerged from cells that express MLH1 as compared to control cells (Figures 4D and 4E). Altogether, these 2 experiments demonstrate that ectopic expression of MLH1 in TS1 cells significantly diminishes the incidences of GF-independent clones and CMP6-resistant subclones.

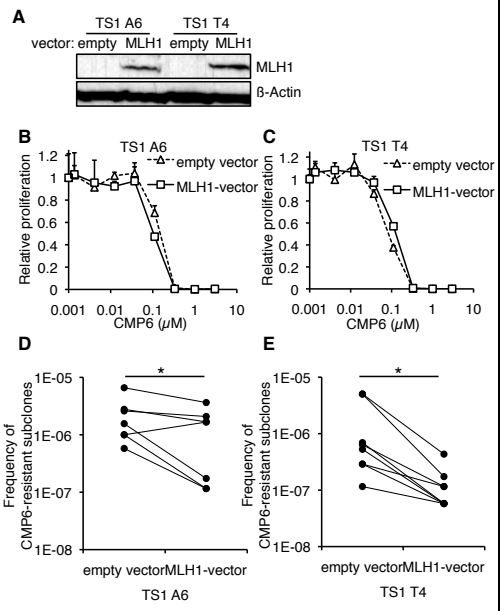


Figure 4. Ectopic MLH1 expression in GF-independent TS1 clones decreases the frequency of CMP6-resistant subclones. (A) MLH1 protein levels were assessed by western blot in TS1 A6 and T4 GF-independent clones electroporated with MLH1 or empty vector. The membrane was probed with anti-β-Actin antibody as loading control. (B) and (C) GF-independent TS1 A6 and T4 clones electroporated with MLH1 or empty vector were seeded in 96-well plates at a density of 1,000 cells/wells in the presence of increasing concentrations of CMP6. After 48 hours of culture, methyl-H^3Thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Thymidine incorporation values were standardized relative to the value in the absence of CMP6. (D) and (E) GF-independent TS1 A6 and T4 clones electroporated with MLH1 or empty vector were plated in the presence of CMP6 (300-600nM) on 96-well plates at a density of 30,000 cells/well. 2-3 weeks later, wells positive for proliferation were visually screened. Graphs represent paired frequency values from 7 and 8 independent selection experiments (each of the 4 electroporated set was at least tested once). Paired Wilcoxon test was performed to determine p-values (*p<0.05)

MLH1 knock-down in TS2 cells promotes oncogenic point mutations in JAK3.

We next sought to determine if the TS2 cell line could give rise to GF-independent clones with acquired mutations in JAK1 and JAK3 upon MLH1 knock-down. To that end, TS2 cells were infected with retroviruses coding for shRNAs against MLH1 or against GFP as a control. In doing so, we obtained 7 paired TS2 bulks coming from 7 independent infection experiments. As presented on figure 5A, shRNA targeting MLH1 successfully silenced 78% of the mRNA expression. Subsequent reduction in MLH1 protein amount was corroborated by western blot on a representative set of cells (Figure 5B).

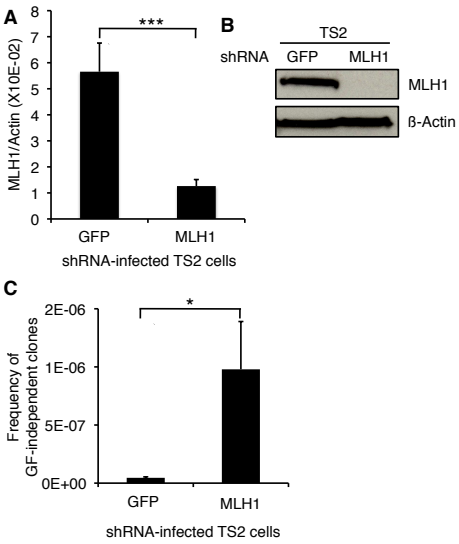


Figure 5. shRNA-mediated MLH1 knock-down in TS2 cells increases the frequency of GF-independent clones. (A) MLH1 mRNA levels assessed by quantitative RT-PCR in TS2 cells infected with retroviruses coding for shRNA against MLH1 or GFP. Histograms represent means +/- SEM of 7 independent infection experiments. Student's t-test was performed to determine p-value. (B) MLH1 protein levels assessed by western blot in shRNA-infected TS2. The membrane was probed with anti-β-Actin antibody as loading control. (C) TS2 cells infected with retroviruses coding for shRNA against MLH1 or GFP were plated in the absence of IL-9 on 96-well plates at a density of 40.000 cells/well. 1-2 weeks later, wells positive for proliferation were visually screened. Histograms represent means +/- SEM of 7 independent selection experiments (each set of infected cells was tested once). Student's t-test was performed to determine p-values (*p<0.05, ***p<0.001).

Altogether, 162 10^6 shRNA-infected TS2 cells were plated in the absence of IL-9 with the hope to select GF-independent clones from MLH1-silenced cells. In line with our expectations, a series of GF-independent clones arose at an average frequency of 1 in a million MLH1-knocked-down TS2 cells. By contrast, only rare (three) GF-independent clones emerged from the control TS2 cells, at an extremely low frequency (< $2X10^{-8}$). 45 GF-independent TS2 clones (including the 3 clones from the control cells) were picked up and screened for mutations in JAK1 and JAK3 (Table 1). 89% (40/45) of them had acquired a mutation in JAK3 whereas none did in JAK1. Altogether, we identified 9 different substitutions affecting 6 conserved residues between mouse and human JAK3. All residues were previously shown to be mutated in GF-independent TS1 clones[23] and for 3 of them (A572, R657 and V674), mutations have been also described in human leukemia[26,27]. The remaining 5 clones displayed no mutation indicating that acquiring a mutation in JAK3 is not the only way for TS2 cells to proliferate independently of growth factor. This contrasts with the TS1 cell line where 100% of GF-independent clones gained a mutation in JAK1 and/or JAK3.
Thus, these data show that selection of GF-independent clones with activating mutations in JAK3 from the MLH1-expressing TS2 cell line is a rare event but its incidence is strongly increased upon MLH1 silencing.

Table 1. JAK3 mutations identified in GF-independent TS2 clones

shRNA	JAK1	JAK3	Number of sequenced clones (N=45)
anti-MLH1	WT	A572V	9
	WT	L586S	6
	WT	R657H	3
	WT	R657C	6
	WT	F666C	2
	WT	V674A	10
	WT	V674G	1
	WT	T848A	1
	WT	WT	4
anti-GFP	WT	R657H	1
	WT	V674F	1
	WT	WT	1

The list of 9 different JAK3 mutations occurring in our collection of 45 sequenced GF-independent TS2 clones (amino acid numbers correspond to human sequence). For each mutation, the absolute frequency is indicated. No mutations were found in JAK1.

TS1 cells and primitive CML cells share a commonmutational profile dominated by T:A>C:G transitions.

Next, we investigated if the loss of MLH1 would be translated into a mutational signature present in our collection of TS1-derived JAK1 and JAK3 mutations. As illustrated on figure 6, nucleotide exchanges in TS1 cells are strongly biased towards T:A>C:G transitions in comparison with the expected pattern of mutations arising spontaneously in normal human cells[28]. Thus, in TS1 cells, the accumulation of T:A>C:G substitutions is favored, potentially as a reflect of MLH1-deficiency or MMR- defect in general. Our data are consistent with studies in bacteria demonstrating that the spectrum of base substitutions is dominated by T:A>C:G mutations in the absence of efficient MMR[29,30].
As already mentioned in the introduction, a fraction of CML patients treated with Imatinib experience disease relapse owing to the emergence of preexisting tumors variants with point mutations in the kinase domain of BCR-ABL. This is somewhat reminiscent of the selection of JAK inhibitor-resistant subclones with acquired mutations in our MLH1-deficient TS1 model. Interestingly, point mutations in BCR-ABL arising in primitive CML cells from Imatinib-naive and -resistant patients are characterized by a predominance of T:A>C:G transitions and an under-representation of C:G>T:A transitions, as a result of a unknown mutator process[31,32]. Knowing this, we compared the profile of nucleotide exchanges in our TS1 model with the one described in CML cells. As depicted on figure 6, TS1 cells and primitive CML cells display an identical mutational pattern, which differs significantly from the standard profile. This analysis indicates that a defect in MLH1 is a potential suspect for the mutator mechanism operative in CML cells that renders them prone to acquire new mutations in BCR-ABL.

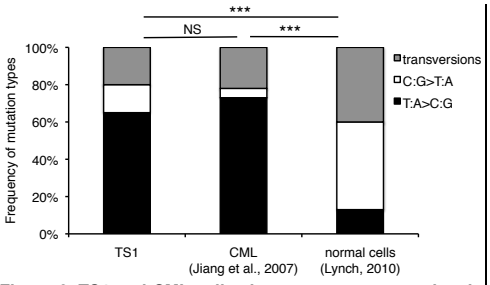


Figure 6. TS1 and CML cells share a common mutational profile distinct from normal cells. The diagrams present the percentages of T:A>C:G and C:G>T:A transitions as well as transversions (substitutions of a purine by a pyrimidine base and conversely) occurring in JAK1 and JAK3 genes for the TS1 cells, in BCR-ABL for CML cells and in unselected regions of the genome for the normal cells (M.Lynch 2010). X^2-test was performed to determine the p-values (***p<0.001; NS, non significant).

MLH1 expression is down-regulated in Imatinib-resistant versus newly diagnosed CML patients.

Given the here above described results, we sought to compare the expression of MLH1 in peripheral blood samples coming from CML relapsed Imatinib-resistant patients and newly diagnosed Imatinib-naïve ones. First, we assessed the mRNA expression of MLH1 in a test cohort including 16 non-paired CML samples (8 at diagnosis and 8 at relapse) from patients referred to the Saint-Luc Hospital in Brussels. For 6 out of the 8 relapsed samples, mutations in BCR-ABL could be detected. In this cohort, MLH1 expression was significantly down-regulated in the samples at relapse as compared to samples at diagnosis (figure 7A, left panel). BCR-ABL/ABL ratios were similar between diagnosis and relapse indicating that CML blood samples from both groups have an equal representation of tumor cells (figure 7A, right panel).

The difference in MLH1 expression was further confirmed on a separate validation cohort coming from the University of Bordeaux comprising 17 diagnosed and 10 relapsed samples from which 6 harbored mutations in BCR-ABL (figure 7B). Thus, selection of Imatinib-resistant tumor subclones in CML patients is accompanied by a simultaneous enrichment of cells with a reduced MLH1 expression, corroborating the clinical significance of our previous in vitro findings.

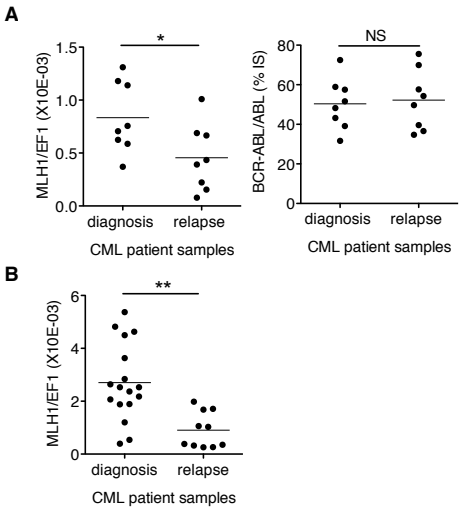


Figure 7. MLH1 mRNA expression is downregulated in Imatinib-resistant versus newly diagnosed CML patient samples. MLH1 mRNA levels assessed by quantitative RT-PCR in CML blood samples taken at diagnosis or at relapse after Imatinib treatment from 2 independent sets of patients, (A, left panel) samples of our test cohort from Saint-Luc Hospital, Brussels (n=8 for both diagnosis and relapse) and (B) samples of the validation cohort from the University of Bordeaux (n=17 for diagnosis, n=10 for relapse). (A, right panel) BCR-ABL/ABL ratios of our test cohort from Saint-Luc Hospital. Horizontal lines represent the mean values. Non-parametric Mann-Whitney one-tailed test was performed to calculate the p-values (*p<0.05, **p<0.01, NS non significant)

DISCUSSION

In this paper, we made the clinically relevant observation that loss of MLH1 expression (or possibly MMR defects in general) continuously promotes the appearance of point mutations in kinases leading to genetically distinct cell variants within a population that are selected by their fitness to adapt upon cytokine deprivation or therapeutic pressure. This finding stems from experiments carried out on the MLH1-deficient TS1 model in which sequential acquisition of activating mutations in JAK1 and JAK3 are required to overcome successively IL-9 withdrawal and exposure to JAK inhibitor. Restoration of MLH1 expression at both stages, IL-9-dependent TS1 cells or GF-independent TS1 clones, significantly reduces the frequencies of further transformed cells with newly acquired JAK mutations, whereas conversely, down-regulation in MLH1-proficient TS2 cells enhances the incidence

of GF-independent clones harboring mutations in JAK3.

At the moment, Ruxolitinib is the only JAK inhibitor that achieved FDA-approval. Although no clinical resistance mediated by acquired mutations have been reported yet upon treatment with Ruxolitinib[33], we believe that our model system would be also relevant in other cancer therapies for which patient-derived drug-resistant mutations are described, as illustrated here for BCR-ABL+ CML. Indeed, it seems very likely that mutations in MMR-deficient cells are not restricted to JAK1 and JAK3 genes but rather occur randomly across the genome. Therefore, extrapolation of our findings to other oncogenes strongly suggests that MLH1-silenced tumors are predisposed to develop secondary resistance against any clinically used targeted therapy.

Imatinib, an ABL1 inhibitor, represents an appropriate clinical illustration of targeted therapy whose effectiveness is limited by the emergence of pre-existing tumor subclones carrying drug-resistant mutations in the kinase domain of BCR-ABL resulting in CML relapse[34]. It has been assumed that the appearance of CML cells harboring such Tyrosine Kinase Inhibitor (TKI)-resistant mutations is fostered by the increased genomic instability in the subset of cells from which the leukemic cells are derived, the so-called leukemia stem cells (LSCs)[35]. This genomic instability in LSCs, manifested as chromosomal aberrations and point mutations (including BCR-ABL mutations)[31], is believed to stem from a combination of increased DNA damages and inefficient repair[36,37]. BCR-ABL itself promotes genetic instability through excessive ROS production[38-40] and inhibition of MLH1-PMS2 dimers assembly[41].

Here, we provide arguments in favor of a down-regulation of MLH1 expression playing a role in genetic instability of LSCs. We show that the pattern of BCR-ABL arising in vivo and in vitro in primitive CD34+ cells from Imatinib-naive and -resistant CML patients strikingly resembles the JAK1 and JAK3 mutations occurring spontaneously in our MLH1-deficient TS1 cells[31]. Indeed, both mutational profiles are characterized by a marked prevalence of T:A>C:G transitions, which is distinct from the natural predominance of C:G>T:A mutations due to spontaneous cytosine deamination[28].

A link between genomic instability and high risk of relapse upon TKI therapy was already evoked by the observation that the overall frequency of BCR-ABL mutations increases with sequential TKI use[42]. In particular, patients who harbor BCR-ABL mutations conferring resistance to first-line inhibitor are more likely to develop additional mutations on second-line or third-line TKI therapy than patients without such mutations[43,44]. This correlation suggests that, in this specific subset of patients, a higher genetic instability boosts spontaneous mutagenesis and fosters the rapid emergence of resistant clones upon treatment. In our paper, we demonstrate that MLH1 expression is significantly down-regulated in leukemic cells from Imatinib-resistant CML patients compared to newly diagnosed ones. This indicates that therapy relapse might be due to selection of a subset of leukemic progenitors with lower MLH1 expression that consequently possess a higher likelihood to acquire additional mutations in BCR-ABL. Our data are further supported by a DNA methylation array study that identified the MLH1 gene as being down-regulated in a Imatinib-resistant subclone of the CML cell line K562[45].

Restoration of MLH1 expression in GF-independent TS1 clones significantly lowers the frequency of JAK inhibitor-resistant subclones. This result suggests that re-activation of the MLH1 locus could promote the effective eradication of MLH1-silenced malignancies. This can be achieved by interfering with epigenetic regulators such as DNA-methyltransferases (DNMT) and histone deacetylases (HDAC). In colon cancers, MLH1 methylation strongly correlates with overexpression of SIRT1, a HDAC frequently up-regulated in many types of solid tumors and hematological malignancies[46]. It has been shown that SIRT1 binds to the hypermethylated promoter of MLH1 and its inactivation causes re-expression of MLH1[47]. This is of particular interest since pharmalogical inhibition of SIRT1 activity blocked acquired resistance to Imatinib in the blast crisis CML cell line KCL-22[48]. However, targeting the epigenetic machinery in order to re-activate the MLH1 locus might fall short from preventing disease relapse. First, as stated by the mutator hypothesis, the accumulation of mutations in genetically unstable cells increases the probability of pre-existing resistant subpopulations within the tumor prior to treatment. In this regard, MLH1 reactivation will have no impact on development of resistance. Second, this approach would be restricted to tumors with epigenetic silencing of MLH1, but ineffective on others harboring inactivating mutations in MLH1 or other MMR components.

An alternative tumor-specific therapeutic strategy would be to exploit the MMR-deficiency by taking advantage of synthetic lethal interactions[49]. Two pathways are synthetic lethal when loss of function of either alone is viable but loss of both causes cell death[50]. For instance, oxidative DNA lesions are mainly removed by the base excision repair pathway (BER), but MMR proteins also contribute to this process as a result of functional redundancy between components of these two DNA-repair systems[51-53]. Therefore, inhibiting or overwhelming the BER machinery would be selectively toxic to MMR-deficient cancer cells through accumulation of oxidative DNA damage while relatively sparing normal MMR-proficient cells[54]. Three different strategies have been explored. First, it has been demonstrated that siRNA-silencing of DNA polymerases specialized in repair of nuclear (POLβ) or mitochondrial (POLγ) oxidative lesions specifically killed cells with deficiency of MSH2 or MLH1 respectively[55]. Albeit potent inhibitors of these polymerases are not currently available, Vitamin K3, better known as menadione, was shown to specifically inhibit POLγ in various cancer cell lines[56]. Interestingly, MLH1-deficient cells were indeed more sensitive to menadione than their MLH1-proficient counterparts[55]. In a second study, silencing of PTEN-induced putative kinase 1 (PINK1) has been identified to elicit synthetic lethality in cells with MSH2, MLH1 or MSH6 dysfunction[57]. PINK1 protects cells from oxidative stress-induced apoptosis and its downregulation resulted in excessive ROS production probably saturating the BER system. To our knowledge, no PINK1 inhibitors have been developed yet. Finally, a comprehensive screening of clinically used chemotherapeutics discovered that cytosine-based nucleoside analogs, such as cytarabine, were selectively lethal to MMR-deficient tumor cells by enhancing levels of intracellular oxidative stress [58].

In conclusion, our results indicate that MLH1-deficient tumors are associated with a high-risk of relapse upon oncogene-inhibitor as monotherapy. Restoring MLH1 function in cancer cells is unlikely to prevent the selection of pre-existing resistant subclones within the tumor cells population. However, the use of described synthetic lethal interactions between MLH1-deficiency and menadione or cytarabine seems more potent in yielding complete and sustained disease remission. Therefore, associating oncogene-targeted therapies with synthetic lethal approaches could represent an attractive strategy in order to specifically and efficiently ablate the MMR-defective cancer cells while avoiding the selection of tumor variants harboring drug-resistant mutations.

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Probe Set ID	Gene Symbol	TS1 / TS2	TS1 A	TS1B	TS2A	TS2B
10569017	Ifitm3	0.01	13.89	26.32	4080.82	3489.12
10346168	Stat4	0.01	21.24	25.17	2402.56	2724.41
10600504	Fundc2	0.01	14.36	12.86	1003.35	1137.31
10593024	Cd3e	0.01	87.25	84.94	6847.35	5929.78
10529711	Clnk	0.01	48.27	40.09	2698.58	3402.52
10358224	Ptprc	0.02	32.03	31.87	2209.53	1989.76
10466127	AW112010	0.02	32.27	30.05	2084.86	1862.02
10574246	Gpr114	0.03	48.93	46.55	1904.56	1804.43
10448202	---	0.03	145.03	151.9	5079.72	4915.08
10463070	Entpd1	0.03	52.46	70.28	1972.67	1825.14
10440340	---	0.03	12.41	10.68	282.91	397.69
10461979	Aldh1a1	0.03	47.37	41.49	1035.42	1525.82
10573451	Syce2	0.04	210.01	126.86	3778.96	4356.5
10548345	Klrk1 /// Klrk1	0.04	19.84	22.41	432.12	584.92
10519983	Fgl2	0.04	47.28	31.04	945.47	921.85
10457644	Cdh2	0.04	39.36	43.16	952.45	975.04
10597288	Mlh1	0.04	21.3	30.65	556.2	632.29
10545101	Hpgds	0.05	35.08	37.9	905.55	669.66
10440131	Gpr15	0.05	33.23	30.02	682.36	531.78
10602277	Luzp4 ///...	20.45	673.77	558.15	31.48	28.76
10602293	Luzp4 /// ...	20.49	660.16	547.01	30.67	28.24
10441787	Airn /// Airn	20.79	1012.31	1537.35	53.85	68.76
10494672	Tbx15	21.51	621.99	490.46	21.62	30.1
10406782	Fam169a	22.29	467.75	738.58	29.47	24.66
10373950	Nipsnap1	22.31	820.89	1160.86	47.31	41.52
10474419	Lgr4	22.72	353.01	349.4	13.85	17.06
10417176	Zic2	24.91	1188.5	1084.22	43.68	47.56
10539873	Gata2	25	2387.56	2922.92	92	120.4
10542965	Sgce	26.2	940.54	720.35	29.83	33.57
10436804	Mrap	26.83	1400.52	1243.41	47.4	51.15
10518358	2610305D13Rik	28.47	261	355.86	12.27	9.4
10403796	Amph	30.07	891.52	981.2	31.41	30.87
10416522	Tsc22d1	30.92	861.23	1361.62	35.76	36.14
10430066	Foxh1	33.47	1963.21	1640.58	61.77	45.9
10606445	Rps6ka6	34.46	677.43	823.37	19.67	23.88
10601844	Bhlhb9	34.59	667.61	784.95	22.78	19.21
10517808	Padi3	35.67	2771.23	2957.54	75.66	84.96
10536294	Peg10	46.33	2057.26	1956.32	42.79	43.84
10602690	Foxr2	47.92	1292.06	1761.86	37.15	26.58
10502240	Npnt	53.1	3453.54	3659.13	69.43	64.53
10416057	Clu	54.59	5990.94	3832.89	94.14	85.81
10580382	Neto2	69.12	909.54	1278.76	18.04	13.62
10522090	Wdr19	77.15	3104.41	3051.51	47.54	32.25
10606178	Xist	96.46	2978.13	2893.62	32.91	27.96

Supplementary table 1. List of genes at least 20X down- or upregulated in TS1 versus TS2 cells.

DISCUSSION

DISCUSSION

The list of hematological malignancies with a significant percentage of cases conveying genetic alterations activating the JAK-STAT pathway is constantly growing. Among them, 42-76% of T-ALL patients present with activating mutations in JAK1, JAK3, TYK2 or IL7Rα, LNK deletions and rare JAK2 fusions, pinpointing a therapeutically targetable "Achilles' heel". As the underlying molecular mechanisms are heterogeneous, exploiting the signaling bottleneck afforded by the limited number of JAK kinases with small molecule TKIs represents an appealing strategy. However,

intra-tumor heterogeneity, dictated by the degree of genetic instability, facilitates rapid adaptation to therapeutic pressures by selection of preexisting cells variant harboring drug-resistant mutations. Consequently, the translation of the oncogene addiction model into clinical use of JAK inhibitors in T-ALL involves a substantial risk for the development of acquired drug resistance phenomena, particularly in the context of a mutator phenotype.

In the present thesis, I took advantage of an original *in vitro* model recapitulating JAK-STAT pathway-driven T-cell transformation in order to evaluate the efficacy of short-term and long-term treatments with JAK inhibitors. At the end of this work, my main observations can be stated as follows: (i) Long-term exposure to JAK inhibitor of cells transformed by activating JAK1 mutations leads to the emergence of resistant cells with acquired activating mutations in JAK3 and *vice versa*; (ii) JAK1 and JAK3 mutants cooperatively activate STAT transcription factors thereby rising the threshold of inhibitor concentration needed to induce cell death; (iii) The acquisition of activating point mutations in JAK kinases is favored by the loss of MLH1; (iv) Substitutions occurring in the context of MLH1-deficiency are strongly biased towards T:A>C:G transitions, so are the point mutations in BCR-ABL arising in primitive CML cells; (v) Cells from Imatinib-resistant CML patients have a lower expression of MLH1 compared to Imatinib-naive patients.

Each of these findings has already been discussed in the manuscripts that compose the results section of this thesis. However, several intriguing questions

raised by our observations deserve deeper considerations. The discussion will address 2 main themes: first the known and yet to be discovered mechanisms underlying the activation of the JAK-STAT pathway in our experimental model, second the origin and significance of the mutational signature observed in TS1 cells in relationship with MMR deficiency.

1. Mechanisms underlying the activation of the JAK-STAT pathway

Why don't we find the same type of CMP6-resistant mutants as previously identified using JAK2 mutagenesis screens?

During my thesis, I identified endogenous mutations that confer resistance to JAK inhibitors by selecting spontaneous preexisting subclones that were able to grow in the presence of CMP6. Unexpectedly, in our TS1 model, resistance to CMP6 was caused by the acquisition of an activating mutation in the JAK partner rather than secondary mutations in the ATP–binding pocket of the JAK mutant that would preclude drug binding. This contrasts with previously reported JAK inhibitor-resistant mutations identified with alternative methods consisting in the screening of randomly mutagenized activated forms of JAK2.

A first screen was performed on BaF3 cells transduced with a library of mutagenized JAK2 V617F cDNA and treated with Ruxolitinib. This study identified 6 secondary mutations located in the kinase domain of JAK2 in Ruxolitinib-resistant cells (M929I/Y931C/G935R/R938L/I960V/E985K)[139]. Using a similar approach, another group isolated 2 identical (Y931C/G935R) and one novel (E864K) mutations in BAF3 cells transduced with a mutagenized JAK2 R683G cDNA library and selected in the presence of another JAK2-inhibitor, BVB-808[140]. In a third study, mutagenesis screen conducted on BaF3 cells expressing the constitutively active TEL-JAK2 fusion protein, revealed several additional mutations (E864K/V881A/N909K/Y918H/R975G/P1057S/R1127K) that could confer resistance to CMP6[141]. Altogether, 13 different inhibitor-resistant substitutions in the kinase domain of JAK2 were described so far. Several affected residues are

located near the ATP-binding pocket, among which 2 (Y931 and G935) were postulated to directly interact with CMP6 based on crystallographic analyses[342]. With the exception of the Y931 residue, which is homologous to the F958 in JAK1 and Y904 in JAK3, there is no evidence that those secondary mutations of JAK2 are gain-of-function mutations.

Surprisingly, except for the Y931 JAK2 residue, none of those 13 resistant mutations were homologous to any of the 15 secondary JAK1 and JAK3 mutations in our collection of inhibitor-selected TS1 subclones. Differences in model settings might explain the discrepancy in the type of mutants identified. JAK2 TKI-resistant mutants were selected from cell lines ectopically expressing the JAK2 cDNA, after *in vitro* random mutagenesis. By contrast, our model involves spontaneous heterozygous mutations of the endogenous JAK1 or JAK3 genes. Since JAK1 and JAK3 function as partners in heterodimeric cytokine-receptor complexes, such as IL-9R or IL-7R, only half of these receptor complexes present in clones with a primary mutation can be associated with an active JAK molecule and are in a partially activated state. As a result of the acquisition of a secondary activating mutation in the JAK partner, half of these receptor complexes can be associated with an active JAK1 or JAK3, but 25% of the receptor complexes can involve both activated kinases, thereby significantly increasing basal activation of the JAK-STAT pathway. By contrast, the aforementioned mutagenesis screens used BAF3 cell-based assays in which active mutant forms of JAK2 were overexpressed, mimicking a homozygous configuration. As JAK2 binds to receptor chains that form homodimers (EPO-R, TPO-R) or heterodimers with another JAK2-binding chain (IL-3R in BAF3 cells), most of the cytokine-receptor complexes are associated with 2 active JAK2 molecules and are in a fully active state. Therefore, secondary gain-of-function mutations in JAK2 are not expected to have a major effect on the activation level of the pathway, which might be already maximal. Instead, acquiring a secondary mutation that would impair drug binding can restore activation of the JAK-STAT pathway in the presence of inhibitor. As a result, the 13 identified JAK2

resistant mutations hinder the effect of the inhibitor without necessarily improving the activity of the kinase.

Does the localization of the secondary resistant mutations support an in trans or in cis mode of JH2-mediated inhibition of JH1 within heterodimeric receptor-JAK complexes?

It is commonly accepted that the function of pseudokinase domain (JH2) of JAKs is to repress the activity of the kinase domain (JH1). However, the molecular mechanism of JH2-mediated suppressive action on the catalytic activity of JH1 is continuously under active debate. Because cytokine receptor complexes crucially comprise at least two subunits each associated with one JAK monomer, both an intermolecular *in trans* or an intramolecular *in cis* mode of inhibition are conceivable and experimental evidences in support of either models have been provided (see the introduction section). However, previous studies used purified recombinant JH1-JH2 peptides or JAK proteins fused to fluorochromes, which mimic conditions distant from the physiological context. The type of JAK1 and JAK3 mutants arising spontaneously in our CMP6-selected TS1 subclones and cooperating in downstream signaling, can inform us about the mode of JH1-JH2 inhibition operative within an unactivated γc-containing heterodimeric receptor.

Based on the observation that cancer-associated JAK mutations map at the JH1-JH2 interface, we can assume that they all share a common mechanism of activation involving the relief of inhibitory interactions between both domains. Given that, in a receptor complex, 2 JAK molecules work as partners, there are 2 inhibitory JH1-JH2 interfaces, either intra- or intermolecular. Consequently, a combination of 2 mutations able to yield a cooperative effect on the overall activity of the complex would be substitutions that abrogate each of the 2 inhibitory interfaces. On the contrary, 2 activating mutations that hit the same JH1-JH2 interface do not cooperatively activate the complex and will not be part of the spectrum of configurations observed in our JAK inhibitor-selected subclones. Figure 11 sketches the possible secondary mutations, starting for example from a

JAK1 JH2 primary mutation of the residue Y652 which participates directly to the interface with JH1[25,343], that would yield or not cooperation according to either an *in cis* or *in trans* mode of inhibition.

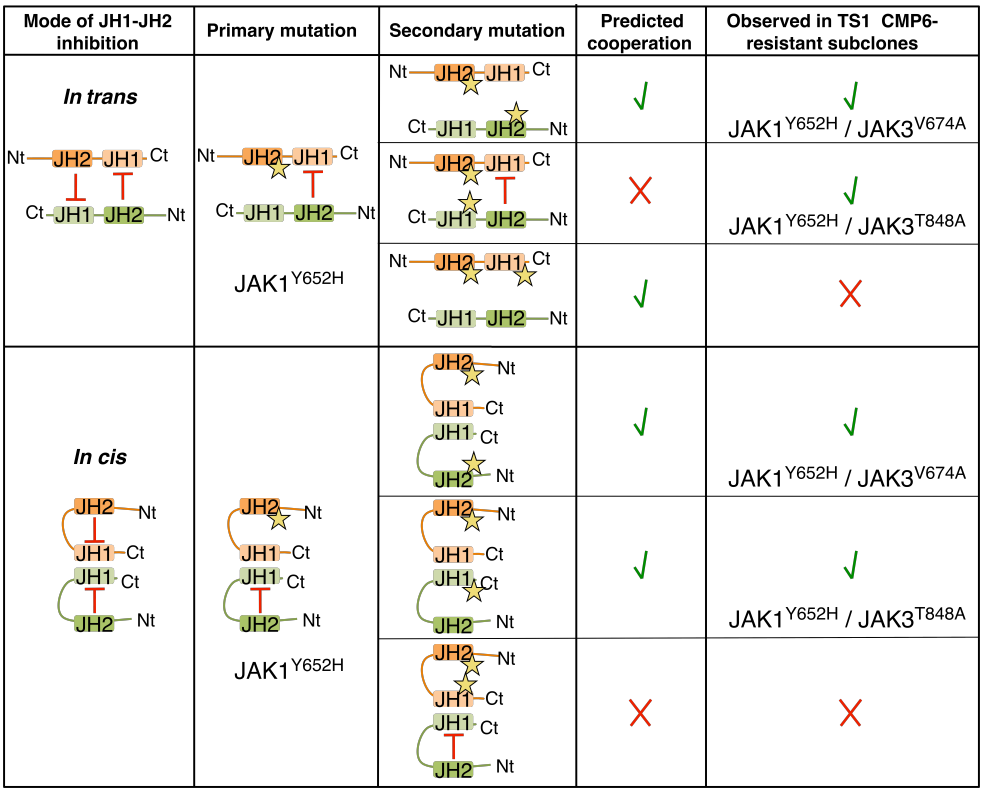


Figure 11. Schematic representation of the different possible concomittant JAK1 and JAK3 mutations and their impact on the overall activity of the heterodimeric receptor complex regarding the 2 proposed models of JH2-mediated inhibition on JH1 catalytic activity. The figure gives an overview of the possible localization of secondary JAK1 and JAK3 mutations starting from a mutation in JAK1 JH2 and indicates if the combination of the 2 substitutions in an *in trans* or *in cis* model can yield a cooperative effect on the overall activity of the receptor complex. Configurations observed in our collection of CMP6-resistant subclones match the ones that yield cooperation in a *in cis* model but not a *in trans* model. The residue JAK1^{Y625} and JAK3^{T848} participate directly to the JH1-JH2 inhibitory interface, JAK3^{V674} is located near the interface. All 3 mutations (JAK1^{Y652H}, JAK3^{V674A} and JAK3 ^{T848A}) are activating and have been associated with human cancers.

In accordance with the *in trans* model, secondary mutations that could provide cooperation with a primary JAK1 JH2 mutation would occur either in JH2 of JAK3 or in JH1 of JAK1, but the latter was not observed in our CMP6-selected

subclones. By contrast, the *in cis* model predicts that the 2 types of cooperating mutations with a JAK1 JH2 mutation can occur in JH1 or JH2 of JAK3, and these are indeed the configurations isolated from our resistant subclones. Furthermore, a JAK1 JH1 mutation would not be cooperative according to this model and is not encountered experimentally leading to the conclusion that our data are consistent with a *in cis* mode of inhibition of JAK1 and JAK3 within a heterodimeric receptor complex.

What are the secondary genetic events occurring in JAK inhibitor-resistant TS1 subclones with no secondary mutations in any JAK kinase?

Altogether, 34% of our JAK inhibitor-resistant TS1 subclones have no secondary mutations in JAK kinases indicating that there exist other ways for transformed cells to grow in the presence of TKI. However, a similar relative shift in sensitivity to JAK inhibitors is observed in both types of selected subclones (mutated and nonmutated), suggesting that the unidentified resistance mechanism causes a boost in the cascade activation to the same extent as cooperation between 2 JAK mutants. As depicted in the first part of the introduction, the molecular alterations underlying JAK-STAT pathway activation in hematological malignancies encompass, besides point mutations in JAK kinases, activating alterations of cytokine receptors or STAT factors, overexpression of cytokine receptors or JAK as well as loss of negative regulators such as tyrosine phosphatases. Each of them represents a potential secondary event that could cooperate with a JAK mutant for downstream signaling, except activating STAT mutations, which are relatively insensitive to JAK inhibition.

Within the framework of the master thesis that she realized in our laboratory, Fanny Lambert, identified the Protein Tyrosine Phosphatase Receptor-type Gamma (PTPRG) as being downregulated in some inhibitor-resistant subclones, albeit without a strict correlation with the absence of secondary JAK mutations. PTPRG is a known tumor suppressor gene whose expression is silenced in 63% of LLA[344] and whose activation has been recently shown to induce JAK2

dephosphorylation[345]. Further studies are needed in order to establish if PTPRG negatively regulates JAK1 or JAK3 activity in which case its absence could boost downstream signals resulting in increased resistance to JAK inhibitors. The fact that loss of PTPRG can arise together with a secondary JAK mutation within the same subclone suggests that, in some instances, multiple genetic events contribute to the resistant phenotype.

Why are TS2 cells transformed by JAK3 mutations but not JAK1?

Strikingly, while activating mutations in JAK1 or JAK3 were identified at similar frequencies in growth factor-independent TS1 clones, only JAK3 mutations were isolated from TS2 cells. This intriguing observation could be the outcome of differentially expressed genes that create distinct cellular contexts within which the presence of a JAK1 or JAK3 active mutant would exert divergent effects on cell proliferation. There are 3 possible options: first, TS2 cells that acquired a JAK3 mutation might have a substantial growth advantage resulting in a relative expansion within the cell population due to natural selection pressure; second, TS2 cells with a mutation in JAK1 might be weeded out owing to a detrimental effect on cell survival; finally, TS2 cells with a mutation in JAK1 might not emerge because the transforming activity of JAK1 mutant is kept silent. Based on micro-array data comparing the transcriptomes of TS1 and TS2 cells, several hypothetical mechanisms consistent with each of the 3 scenarios can be proposed.

In the first case where JAK3 mutants are positively selected, variations in the expression of JAK1-binding receptor chains that associate with γc bound to JAK3 can represent a plausible explanation. Indeed, while the IL-9Rα and IL-4Rα are present at similar levels in TS1 and TS2, the expression of IL-7Rα, IL-2Rβ and IL-2Rα are restricted to TS2 cells. JAK3 mutants might possibly produce higher pro-proliferative signals when it is part of an IL-7R or IL-2R complex rather than IL-9R or IL-4R.

The second alternative hypothesis postulates that JAK1 mutants arising in a TS2 cellular context are subjected to negative selection because it generates

unfavorable signaling. JAK1 participates in the intracellular transduction of type I interferons (IFN), which are known to exert an anti-proliferative activity on various cell types. It has been previously shown that some JAK1 mutants potentiate the cellular response to type I IFNs rendering cells hypersensitive [75,78]. TS2 cells might be intolerant to the presence of JAK1 mutants that would exacerbate constitutive type I IFNs signaling up to a threshold incompatible with cell survival.

The third option states that the transforming potency of JAK1 mutants is repressed in TS2 cells and therefore does not confer growth supremacy over neighboring cells. One possible mechanism implicates PTPRC, a protein tyrosine phosphatase that negatively regulates JAK-STAT signaling downstream of cytokine receptors[346,347] and which is present in TS2 cells but absent in TS1 cells. It is conceivable that PTPRC presents variable substrate affinity among the JAK family members and that, in a γc-containing heterodimeric receptor complex, its catalytic activity is mainly focused on JAK1 rather than JAK3. Therefore, as TS2 cells express high levels of PTPRC and TS1 cells do not, JAK1 mutants can only manifest in a TS1 cellular context.

2. MMR-deficiency and mutational signature

Can the mutational bias towards T:A>C:G transitions observed in our TS1 model be attributed to the deficiency in MMR?

The molecular fingerprint of MMR-deficient tumors is shaped by DNA replication errors that persist in defiance of the sequential operation of 2 evolutionary conserved processes: the selectivity of replicative DNA polymerases for incorporating complementary bases into Watson-Crick pairs and their proofreading activity[348]. The accuracy of polymerases relies on the thermodynamic stability afforded by the formation of 2 or 3 hydrogen bonds between the incoming nucleotide and the first non-paired base of the template strand as well as spatial restrictions within their active site[349]. High-fidelity polymerases are intolerant to helix distortions caused by the incorporation of non-complementary base pairs and have inbuilt proofreading activity that precludes further extension unless the

terminal mispaired nucleotide is excised and replaced. Even this, however, is not sufficient to prevent mismatch formation, the most frequent being the wobble T:G mispair that is stabilized by 2 inter-base hydrogen bonds and brings about only a slight distortion of the DNA helix from which polymerases are able to extend with the least difficulty[349,350]. If not repaired by the MMR system, the T:G mispairs gives rise to transitions (i.e. purine-to-purine or pyrimidine-to-pyrimidine substitutions) in 50% of progeny.

Interestingly, among the different possible mismatches, MutS dimers bind with the highest affinity to T:G heteroduplexes[351-353]. Thus, the most commonly formed mispair corresponds to the one best recognized by MutS proteins indicating that the MMR system has evolved to efficiently counteract post-replicative mismatches arising at the highest rate, in a reciprocity between generating and correcting errors[354-356]. Consequently, in the absence of functional MMR, a general increase in the relative frequency of transitions versus transversions is observed in bacteria[357,358], yeast[359,360] and human cells[267,361] as a result of highly prevalent uncorrected T:G mispairs. In normal human cells, the transitions versus transversions ratio is 1.7[362], in MMR-deficient tumors this ratio ranges from 2.35[267] to 2.85[361] and in our TS1 model it reaches 4.

In almost all organisms, the pattern of spontaneous mutations is dominated by C:G>T:A transitions resulting from the natural deamination of C to U (and of methylated C to T at CpG sites). Strikingly, in our TS1 model and in 2 different MMR-deficient bacteria strains (one lacking MutS[358], the other lacking MutL[357]), the expected trend is inverted in favor of T:A>C:G. It is worth noting that the bias observed in TS1 is distinct from the one induced in the context of mutated proofreading-deficient polymerases ε or δ, characterized by a particular increase in C:G>A:T transversions[288,363]. This suggests that an enhanced frequency of T:A>C:G transitions specifically reflects a defect in MMR.

As already mentioned, unrepaired T:G mispairs generate transitions in either directions according to the symmetry of the error. The incorporation of a dTTP opposite to a template G (G:dTTP) gives rise to C:G>T:A while the other way round (T:dGTP) brings about T:A>C:G transitions. Based on the observed bias in our TS1

model, T:dGTP mispairs seem to be more frequent or less corrected relative to G:dTTP in MMR-deficient cell genome as a result of a still elusive mechanism.

Since many DNA repair pathways crosstalk with each other, it is conceivable that, in the absence of MMR, an alternative system works as compensatory backup[364]. The functional interplay of MMR with BER elects this latter as prime candidate for this job. Indeed, BER has been shown to participate in repair of engineered T:G mismatches but might not completely counterbalance the lack of MMR as it does not discriminate between parental and daughter strands[365]. In 75% of cases, T:G mispairs were reverted to C:G indicating that the G-containing strand was preferentially used as template, thereby inducing a mutational bias towards T:A>C:G transitions. A second hypothesis would postulate that compensatory repair involves the action of low-fidelity DNA polymerases[366]. Several of those error-prone polymerases such as polη[367], polλ[368] and polι[369,370] frequently misincorporate a dGTP opposite to template T thereby increasing the level of T:dGTP mismatches[371] (Figure 12).

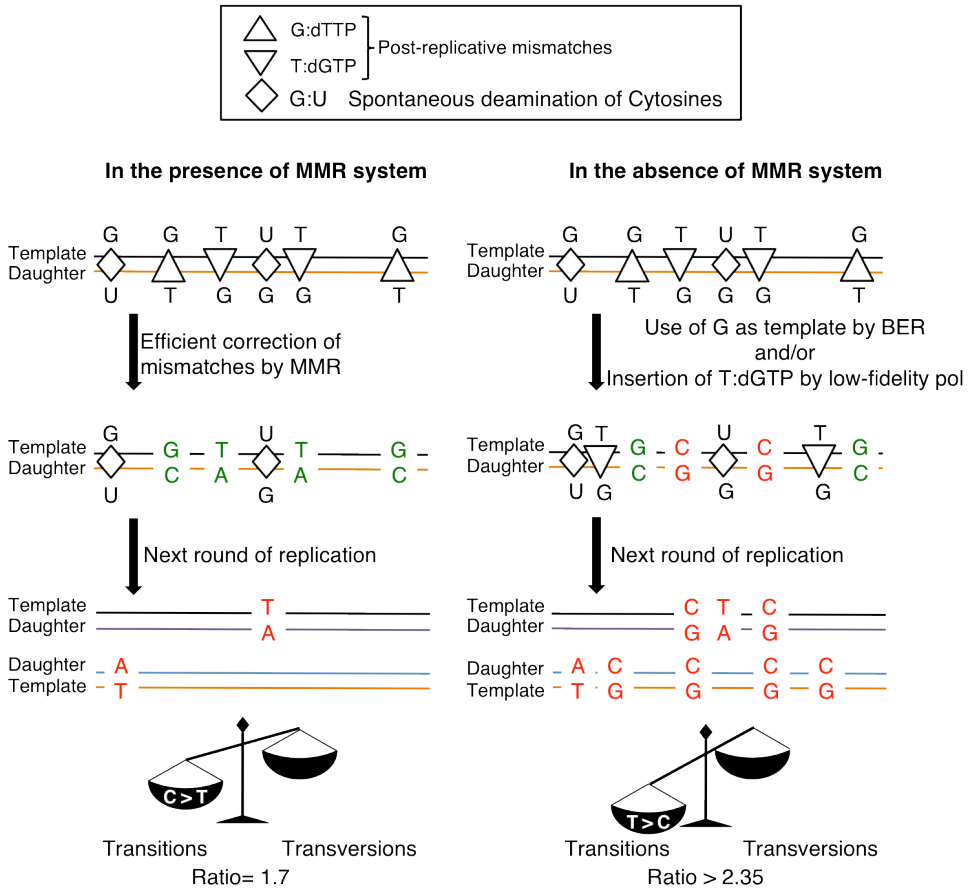


Figure 12. Proposed model for the increased frequency of T>C transitions in the absence of efficient MMR. T:G mispairs (triangles) are the most frequent post-replicative errors. In the presence of MMR, T:G are efficiently corrected and the mutation pattern is dominated by C>T transitions resulting from the spontaneous deamination of cytosines. In the absence of MMR, T:G are handled by a compensatory backup system that either preferentially converts T:G into C:G or involves the action of low-fidelity polymerases that misincorporate G opposite to template T. In this case, the ratio of transitions versus transversions is exacerbated due to the considerable increase in T>C substitutions.

Is the mutational signature identified in our TS1 model relevant for MMR-deficient human cancers?

The collective somatic mutations observed in a cancer are the outcome of multiple mutagenic processes, including DNA damages and inefficient or error-prone repair, that have been operative over a lifetime, each of which leaving a characteristic imprint on the genome[372]. The proportion of the total mutation load enforced by

each mutagenic process correlates with its intensity and duration of exposure throughout the biological history of the cancer[372]. According to the here above proposed model, the absence of efficient MMR can account for the particular pattern of mutations dominated by T>C transitions in TS1 cells. Consequently, it was expected that computational analysis aiming at deciphering molecular imprints of mutational processes in cancers would yield a similar molecular signature in tumors lacking MMR. Nevertheless, such bias towards T>C transitions has not been reported in MLH1-silenced colon and endometrial cancers[248,373]. Rather, the mutational signature associated with low expression of MLH1 was characterized by a predominance of C>T transitions and C>A transversions[248,373].

Differences in time scales and degree of heterogeneity within the studied cell population might underlie the discrepancy between human tumors and *in vitro* TS1 model. First, contrasting with mutations arising over a relatively short interval in TS1 cells, the genetic alterations composing the mutational landscape of human cancers are accumulated over a lifetime and a substantial part of them likely occurred prior to the inactivation of MMR. Second, patient tumors comprise a mix of cell subpopulations, which might be exposed differentially to various mutagens, possess variable residual MMR activity or convey dysfunctions of multiple DNA repair proteins. For instance, a certain number of colon and endometrial cancers are known to bear loss-of-proofreading mutations in POLE additionally to MLH1 silencing. Therefore, the final mutational portrait of such tumors represents a complex composite of different signatures[374] within which the T>C transitions are overwhelmed by other types of substitutions, while the mutational pattern in less heterogeneous populations such as TS1 cell cultures might more faithfully and specifically reflects MMR deficiency.

Finally, it should be noted that although several of the actual 21 validated mutational signatures[248,372] have been attributed to a specific mutagenic process, the mechanisms underlying other recognized signatures (among which some featuring a predominance of T>C mutations) remain to be undertsood[248,373]. Furthermore, it is likely that additional signatures of cryptic origin will be extracted

from the growing number of sequenced cancer genomes. In the future, we predict that a signature characterized by an overrepresentation of T>C transitions will be identified as being the outcome of inefficient MMR.

CONCLUSIONS

CONCLUSIONS

Over the past decade, advances in the understanding of cancer etiology fuelled efforts to develop pharmaceuticals targeting signaling proteins that acquired tumor-promoting functions, especially mutated tyrosine kinases, and some of these small molecule inhibitors are now part of current treatment protocols. However, according to experiences in the clinic, it has become clear that the use of TKIs in oncokinase-driven malignancies will not be curative as stand-alone therapies owing to the emergence of diverse acquired resistance phenomena that cause disease to relapse.

In this thesis, we described a new mechanism of resistance to JAK inhibitors and it is likely that other strategies implemented by cancer cells to overcome TKI will emerge with the growing number of treated patients and the enlargement of clinical indications. Avoiding those resistance phenomena therefore represents a major challenge.

In this context, the future of targeted therapies might reside in the alliance of drugs targeting effectors of parallel signaling cascades, such as inhibitors of JAK, PI3K and MAPK that act synergistically and involve a lower risk of circumvention by single-target mutations or the activation of bypass pathways. However, such combinatory therapeutic approaches could be too simplistic to face a more complex situation. Kinase mutations are generally associated with recurrent additional genetic lesions affecting transcription factors or epigenetic modifiers that can modulate cancer progression and drug sensitivity. Attaining the optimal clinical efficacy of TKI would probably require the entire molecular characterization of the cancer in order to predict the rationale drug combination with the highest likelihood of success[157]. The advent of low-cost NGS techniques is expected to enable such "personalized medecine" by carrying out the inventory of oncogenic variants present in a given tumor[375]. However, tumors are constituted of genetically distinct cell variants and interpreting the mutational identity card of a cancer as a homogenous population could generate a biased picture and compromise prediction of therapeutic response.

Conclusions

Nevertheless, the analysis of the global mutational portrait, regardless of whether the alterations are drivers or passengers, can unveil particular signatures attesting the abrogation of specific DNA repair pathway(s). In this thesis, we demonstrate that loss of MLH1 expression not only raises the level of point mutations but also imprints their pattern of substitutions with a remarkable increase in T>C transitions. Knowledge of the invalidated repair system in a given tumor, such as MMR deficiency, could assist clinical decisions regarding potential sensitivity to therapeutics and thus provide a more successful path for cancer disease control from 2 sides[372]. First, information about MMR status would enable the stratification of patients with a higher probability of primary refractoriness to classical DNA-damaging agents or acquired resistance to targeted therapy that would require special follow-up care. Second, it could pinpoint tumors eligible for synthetic lethal approaches where the absence of MMR is specifically exploited.

This latter therapeutic strategy is made possible by the fact that DNA repair operates as a network of pathways sharing functional overlap rather than isolated entities so that cancer cells lacking one system often become reliant on an alternative backup[376]. For instance, MMR system is involved in the non-canonical processing of oxidized bases and inhibiting or overwhelming BER that classically resolves these lesions is selectively toxic for MMR-deficient tumor cells while sparing normal MMR-proficient cells. An on-going phase II clinical trial is evaluating the potential of methotrexate, an inducer of oxidative DNA damages, to translate into clinically efficient treatment options for MSH2-deficient metastatic colorectal cancers (NCT00952016)[377].

The second aspect of the paradox related to MMR deficiency being both a strength and a weakness for a cancer cell, concerns the boost in immunogenicity resulting from the generation of tumor-specific antigens due to increased somatic mutations. In this regard, benefit can be gained by using inhibitors of programmed death-1 (PD-1), a repressor of TH1 cytotoxic immune responses. A phase II study has been conducted on patients suffering from progressive metastatic carcinoma (colon cancer and melanoma) with or without MMR deficiency and concluded that MMR-

deficient tumors were indeed more responsive to PD-1 blockade than MMR-proficient counterparts (NCT01876511)[378].

In conclusion, switching-off the oncogenic pathway by targeted therapies involves the risk of accelerating and worsening the natural history of the disease by selecting tumor cell variants with additional genetic lesions. Moreover, repeated rounds of selection, for example by sequential use of small molecule inhibitors, are likely to promote progressive enrichment in DNA repair deficiencies that will entail increased mutagenesis and tumor heterogeneity. However, this higher plasticity evolves at the cost of supplementary vulnerabilities that can be turned to account by synthetic lethality or anti-tumor immune surveillance. Therefore, the comprehensive consideration of cancers by integrating their targetable advantageous properties as well as exploitable shortcomings should serve as guidelines for the design of future clinical trials.

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ANNEX

ANNEX

Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors

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ABSTRACT

Background
Activating mutations in JAK1 and JAK2 have been described in patients with various hematologic malignancies including acute lymphoblastic leukemia and myeloproliferative neoplasms, leading to clinical trials with JAK inhibitors. While there has been a tremendous effort towards the development of specific JAK inhibitors, mutations conferring resistance to such drugs have not yet been observed.

Design and Methods
Taking advantage of a model of spontaneous cellular transformation, we sequenced JAK1 in selected tumorigenic BaF3 clones and identified 25 *de novo* JAK1 activating mutations, including 5 mutations already described in human leukemias. We further used this library of JAK1 mutation-positive cell lines to assess their sensitivity to ATP-competitive inhibitors.

Results
While most JAK1 mutants were sensitive to ATP-competitive JAK inhibitors, mutations targeting Phe958 and Pro960 in the hinge region of the kinase domain rendered JAK1 constitutively active but also resistant to all tested JAK inhibitors. Furthermore, mutation of the homologous Tyr931 in JAK2 wild-type or JAK2 V617F mutant found in patients with myeloproliferative neoplasms also conferred resistance to JAK inhibitors, such as INCB018424, which is currently in clinical use.

Conclusions
Our data indicate that some activating mutations not only promote autonomous cell proliferation but also confer resistance to ATP-competitive inhibitors. *In vivo*, such a mutation can potentially occur as primary JAK-activating mutations but also as secondary mutations combining oncogenicity with drug resistance.

Key words: JAK, tyrosine kinase, mutations, inhibitors, STAT.

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Annex

Introduction

Persistent activation of signal transducer and activator of transcription (STAT) factors is characteristic of many types of leukemia and solid tumors. In normal cells, cytokine-induced activation of STAT proteins provides the stimulus for proliferation, survival, self-renewal, differentiation and functional activation. In leukemic cells, like in other cancers, STAT proteins are frequently constitutively activated in the absence of cytokine stimulation, providing malignant cells with persistent signals and self-sufficiency for survival and proliferation.[1]

The most common mechanism for constitutive activation and phosphorylation of STAT factors is the dysregulation of tyrosine kinases. In 2005, the identification of an activating mutation in JAK2 (the V617F mutation) as a STAT5-activating and disease-causing genetic alteration in a significant proportion of patients with myeloproliferative neoplasms (MPNs) has emphasized the oncogenic role of the JAK tyrosine kinases in hematologic malignancies.[2-5] JAK2 is a member of the Janus tyrosine kinase family comprising three other mammalian non-receptor tyrosine kinases (JAK1, JAK3 and TYK2) that associate with cytokine receptors lacking intrinsic kinase activity to mediate cytokine-induced signal transduction and activation of STAT transcription factors.[6] All JAKs share a similar protein structure and contain a tyrosine kinase domain at the C-terminus flanked by a catalytically inactive pseudokinase domain with kinase-regulatory activity, by an atypical SH2 domain and by a FERM domain that mediates association to the membrane-proximal region of the cytokine receptors.[7,8] Soon after the discovery of JAK2 V617F, we and others described that activating JAK1 mutations are relatively common in adult patients with T-cell acute lymphoblastic leukemia (ALL) and participate in ALL development allowing for constitutive activation of STAT5.[9-11] Several STAT5-activating JAK1 mutations were also reported in AML and breast cancer patients.[10]

Because of its central role in the pathophysiology of MPNs, the JAK2 V617F is an attractive therapeutic target. Several JAK2 inhibitors are now under development or/and undergoing clinical trials for MPNs. Some of these JAK2 inhibitors were also shown to be remarkably active on JAK1 and have, therefore, a strong therapeutic potential for JAK1 mutation-positive patients. One of the most promising JAK1/JAK2 inhibitors is INCB018424,[12] currently in phase III clinical trials for MPNs. The major drawback of tyrosine kinase inhibitor therapy is the development of secondary resistance caused by the acquisition of new mutations. The best example of this scenario comes from BCR-ABL positive neoplasms. Here patients treated with imatinib become resistant because of the acquisition of mutations in the ABL kinase domain.[13]

In our laboratory, we have previously described an *in vitro* model of spontaneous transformation of the IL-3-dependent hematopoietic BaF3 cell line towards growth factor-independent tumorigenic clones with constitutive STAT5 activation.[14] This model was developed from the study of BaF3 cells transfected with an IL-9Rα mutant lacking the STAT-recruiting site (BaF3 phe116). This BaF3 phe116 almost completely fails to proliferate and activate STATs in response to IL-9.[15] However, upon prolonged culture with IL-9, a small number of cells manage to survive and even proliferate, allowing an IL-9-dependent cell line (BaF3 phe116/9) to be selected.[14] In contrast to parental BaF3 phe116 cells, those IL-9-selected cells could progress to autonomous cells (BaF3 Aut) after a second selection step in the absence of cytokine. These autonomous cells show a cytokine-independent activation of JAK1 and STAT5 and are highly tumorigenic when injected in mice, which is not the case for parental BaF3 phe116 and BaF3 phe116/9.[14,16] We previously showed that upregulation of the endogenous JAK1 gene was associated with the first step of transformation, namely increased sensitivity of BaF3 phe116 cells to IL-9, and promotion of the second step of transformation, namely progression towards cytokine-independent BaF3 autonomous cells.[16]

In this study, we show that 80% of the autonomous BaF3 clones, selected in our *in vitro* model, acquired activating point mutations in the kinase or pseudokinase domain of JAK1. These JAK1 mutations provide cells with tumorigenic potential by inducing constitutive activation of the JAK-STAT pathway, which supports their autonomous proliferation. We took advantage of this collection of JAK1 mutation-positive autonomous cell lines to study the sensitivity of different JAK1 mutations to JAK inhibitors. For the first time, we report that mutations of the Phe958 and Pro960 not only constitutively activate JAK1, but also render the mutated JAK1 protein resistant to ATP-competitive inhibitors. The homologous mutation in JAK2, namely Y931C, also renders JAK2 wild-type or V617F mutant resistant to all tested ATP-competitive inhibitors.

Design and Methods

Cell culture and cytokines

BaF3 mouse hematopoietic pro-B cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and IL-3 (150 U/mL), which was produced by transfected CHO cells. Recombinant human IL-9 was produced in the baculovirus system and purified by affinity chromatography in our laboratory. The generation of BaF3 phe116 as well as BaF3 phe116/9 cells and the selection of autonomous cells has been previously described.[14] The frequency of autonomous cells was assessed as previously described.[14] IL-9-selected BaF3 phe116/9 and non-selected BaF3 phe116 cells were grown in the presence of IL-3, while autonomous clones were selected and subsequently amplified in the absence of IL-3.

RNA extraction, cDNA synthesis, PCR and sequencing

Total RNA was isolated from 10^6 IL-3 dependent BaF3 phe116, BaF3 phe116/9 or autonomous BaF3 (BaF3 Aut) clones using TriPure reagent (Roche) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA with an oligo (dT) primer (Roche) and M-MLV RT (Invitrogen). PCR amplification was performed from cDNA corresponding to 20 ng of total RNA at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min with a total of 39 cycles. Depending on the region of JAK1 to be sequenced, different sets of primers were used to amplify and sequence JAK1 PCR product (available upon request). PCR product was purified using Chromaspin technology (Clontech): 50-100 ng of PCR product was used for sequencing with the DYEnamic ET Dye Terminator Kit (Amersham Biosciences) according to the manufacturer's instructions. Two independent PCR reactions were performed to rule out the possibility of Taq-induced mutations during amplification.

Interestingly, all the nucleotides mutated in the murine JAK1 sequence were conserved in the human JAK1 sequence (*data not shown*). Therefore, the numbering of mutated amino acids was based on the human JAK1 sequence, allowing currently used nomenclature for JAK1 protein mutations in literature to be adopted and to localize our JAK1 mutations in the previously described three-dimensional structure model of human JAK1 protein.[9]

Reverse transcription-quantitative PCR (RT-PCR)

Reverse transcription was performed as described above. Quantitative PCR reactions were performed using primer sets corresponding to murine JAK1 or murine Actin with qPCR™ Mastermix for SYBR Green I (Eurogentec). The sequences of primers (final concentration 300 nM) were: mJAK1 5'-GGAGTGCAGTATCTCTCCTCTCT-3' (forward) and 5'-CCATGCCCAGGCACTCATTTTCA-3' (reverse); mActin 5'-GCTGGAAGGTGGACAGTGAG-3' (forward), 5'-CTCTGGCTCCTAGCACCATGAAG-3' (reverse). To amplify corresponding cDNA, PCR conditions were used as previously described.[17,18] Results were analyzed using MyiQ software. Starting quantity of the transcripts was calculated using standard curves from a cDNA clone.

Plasmid construction, stable DNA transfections and analysis of transfected cells

All JAK1 and JAK2 mutants were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene) and subcloned into the pMX-GFP (for JAK1) or pMEGIX-GFP (for JAK2) biscistronic retroviral vector upstream of the IRES as previously described.[17-19] The mutated JAK1 and JAK2 constructs were verified by sequencing the full-length JAK1 and JAK2 using DYEnamic ET Dye Terminator Kit (Amersham Biosciences). For stable transduction, typically 0.5×10^6 BaF3 cells were infected by retroviruses produced by BOSC packaging cells using a previously described standardized protocol.[20] Cells were subsequently sorted by FACS according to equal levels of GFP.

Western blots

For Western blot analysis, 10^6 BaF3 cells were starved for 4 h and lysed in 250 μL of Laemmli buffer (BioRad). We separated proteins using 12% pre-cast Tris-Glycine gels (Invitrogen) and transferred them to nitrocellulose membranes (Hybond-C, Amersham). Phosphorylation of signaling proteins was investigated using the following phospho-specific antibodies from Cell Signaling Technology: anti-pY1034/1035 JAK1 (#3331S), anti-pY1007/1008 JAK2 (#3771S), anti-pY705 STAT3 (#9131S), anti-pY694 STAT5 (#9351S) and anti-p44/42 MAPK (#9101L). Blots were re-probed with anti-JAK1 (#3332), anti-JAK2 (#24B11) (Cell Signaling Technology, Beverly, MA, USA), anti-STAT5 (Santa Cruz) or anti-β-Actin (Sigma) antibodies, as control. All the antibodies were used at dilutions recommended by the manufacturer.

JAK inhibitors and proliferation assay

To test the effect of JAK inhibitors, 10,000 stably-transduced or JAK1 mutation-acquired autonomous BaF3 cells were seeded in 96-well plates in the presence of JAK inhibitors CMP6 or INCB018424 at different concentrations (10 - 0.001 nM) and DMSO as control. After 48 h, tritiated thymidine was added to the cells for 4 h. Cells were then collected on microfiltered plates, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT, USA).

JAK1 structures

The three-dimensional structural model of the kinase and pseudokinase domain of JAK1 has been previously described[9] and was obtained by employing Deep View software and the Swiss Model server after manual regulation of the best alignment. Structural data from recently solved crystal structure of JAK1 kinase domain in complex with CPM, an ATP-competitive inhibitor (PDB 3EYH),[21] were used to analyze more precisely the impact of the kinase domain-located JAK1 mutations. Molecular graphic images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, USA.[22]

Statistical analysis

Significant differences were determined using InStat software. The mean of 2 groups was compared with Student's t-test. Normality tests were used to test the assumption of a normal distribution. Mean values ± SEM are shown.

Results

De novo *mutations in kinase and pseudokinase domain of JAK1 spontaneously occur during selection of BaF3 autonomous clones*

The transformation of BaF3 phe116 into autonomous tumorigenic clones is a two-step model of tumorigenesis. The first step requires spontaneous upregulation of JAK1, while the mechanism involved in the second step of transformation is not known.[16] As constitutive phosphorylation of JAK1 was detectable in autonomous clones,[14] we systematically sequenced JAK1 from autonomous clones and found that the vast majority of them (139 of 164) were heterozygous for a single *de novo* mutation. Altogether, 25 different missense mutations were identified as listed in the *Online Supplementary Table S1* and *Online Supplementary Figure S1*. Among the 25 different mutations identified, 12 affect residues located in the pseudokinase domain and 13 in the kinase domain (Figure 1A and B). Most of the remaining JAK1 mutation-negative clones have acquired the IL-3 autocrine loop (*data not shown*). An unknown genetic or epigenic event remains to be identified in 3 last autonomous clones that are JAK1 mutation-negative and IL-3 autocrine loop-negative.

To better highlight the localization of mutated residues, we took advantage of the three-dimensional model structure of kinase (JH1) and pseudokinase (JH2) domain of JAK1 previously described.[9] As shown in Figure 1B, half of the affected residues (6 of 12 *de novo* mutations) of the pseudokinase domain (F635V, S646F, Y652H and V658I/L/F), including 3 mutations (S646F, Y652H and V658F) that were previously identified in T-ALL patients,[10,11,23] are located within the pocket formed between the JH2 αC helix and an adjacent loop comprising Val658 and Tyr652 residues. These two regions of the JH2 domain were predicted to interact with the activation loop of the kinase domain in the active conformation.[24] The enrichment of this region in activating mutations suggests that this area represents a hotspot for mutations that might share a common mechanism to activate JAK1. Three of the JH1-located mutations (K1026E, Y1035C and S1043I) are also targeting the activation loop of the JH1 domain that is part of the interface between the kinase (JH1) and the pseudokinase (JH2) domain, further sup-

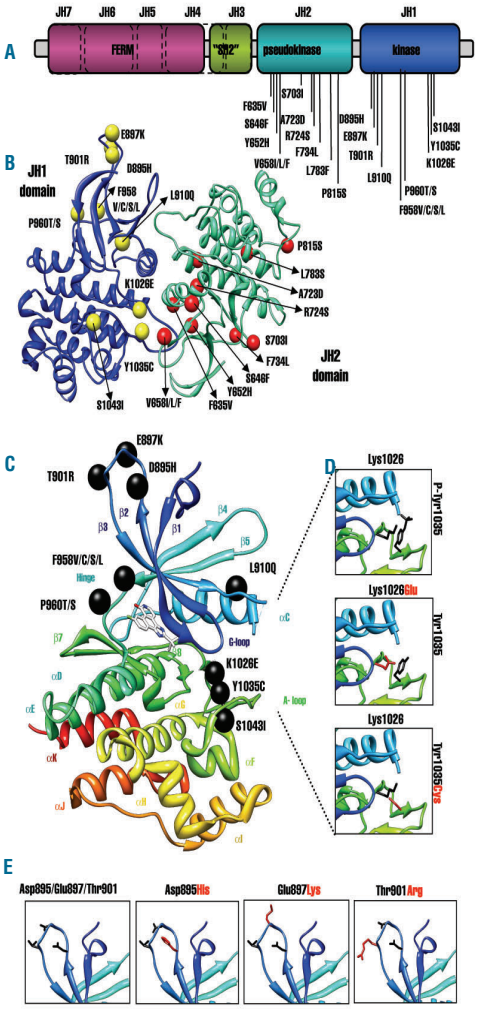


Figure 1. *De novo* mutations in kinase and pseudokinase domains of JAK1 spontaneously occur during selection of BaF3 autonomous clones. (A) Localization of 25 identified missense point mutations affecting 19 residues in the schematic structure of JAK1 with its functional domains and (B) in the three-dimensional modeled structure of kinase and pseudokinase domain published by Flex[3]. Ten affected residues (red balls) in pseudokinase (in green) and 9 (yellow balls) in kinase domain (in blue) are shown. (C) Localization of 9 affected residues (black balls) in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 (PDB 3EYH).[21] The JAK inhibitor is presented in white using a ball-and-stick representation. (D) Magnification of structure of the activation loop showing the side chains of the residues at positions 1026 and 1035 for the wild-type JAK1 (top panel), the K1026E mutant (middle panel), and the Y1035C mutant (lower panel). (E) Magnification of the β2/β3 linking loop highlighting the side chains of the residues at positions 895, 897 and 901 for the wild-type JAK1 (left panel), the Asp895His mutant (left middle panel), the Glu897Lys (right middle panel) and the Thr901Arg mutant (right panel).

porting this hypothesis.

For the mutations located in the JH1 kinase domain, we took advantage of the recently solved crystal structure of the JAK1 JH1 domain to detail their localization.[21] As shown in Figure 1C, D and E, from 13 JH1-located mutations, 3 (K1026E, Y1035C and S1043I) are located in the activation loop (A-loop), 4 mutations affect the same residue (F958V, F958C, F958S, F958L) in the hinge region of the kinase domain at the entry of the ATP-binding pocket, 3 others (D895H, E897K and T901R) are located at the top of the kinase domain in the loop formed between two antiparallel β-strands (β2 and β3) and one mutation affects the loop formed between the β-strand-3 (β3) and the αC helix of the JH1 domain (L910Q). Altogether, beside the mutations targeting the JH1/JH2 interface, the other activating mutations found in this study point to two other hotspots for the regulation of JAK enzymatic activity: the hinge region and in the loop formed between β2- and β3-strand in the kinase domain. Moreover, structural modeling of these hotspots also suggests that each of them might have a distinct molecular mechanism for kinase activation.[25]

JAK1 mutations spontaneously occurring in autonomous BaF3 cells are JAK-STAT pathway activating mutations

We previously showed that progression of BaF3 phe116/9 cells toward cytokine-independence was associated with constitutive activation of JAK1 and STAT5.[14] We, therefore, examined whether all JAK1-mutated autonomous clones (BaF3 Aut) showed a similar signaling profile. We chose one representative JAK1 mutation-positive autonomous BaF3 clone for each of the 25 JAK1 mutations and performed Western blot analysis to assess the activation status of JAK1, STAT5 and ERK1/2. As shown in *Online Supplementary Figure S2A*, none of these proteins were phosphorylated in BaF3 phe116 or IL-9-selected BaF3 phe116/9 in the absence of cytokine. In contrast, all autonomous BaF3 clones showed constitutive activation of STAT5 and, for most of them, constitutive phosphorylation of JAK1 and ERK1/2 was also detected, confirming that all JAK1-mutated autonomous clones harbored constitutive activation of the JAK-STAT and the MAP kinase pathways. As expected, the total level of JAK1 was 2-3 times higher in BaF3 phe116/9 and in autonomous clones as a consequence of the first selection step, which consists of spontaneous JAK1 upregulation.[16] To confirm that the acquisition of mutations in JAK1 is responsible for autonomous BaF3 cell proliferation, we produced mutated JAK1 constructs for the first 13 of the 25 JAK1 mutations that we had found. We transduced parental BaF3 cells with either mutated or wild-type (WT) JAK1. In contrast to cells stably transduced with WT JAK1, which quickly died after IL-3 starvation, all JAK1 mutated constructs were able to promote the autonomous growth of BaF3 cells (*data not shown*). Moreover, similarly to autonomous BaF3 harboring spontaneously acquired mutations, all JAK1 mutants induced the constitutive activation of STAT5 and MAP kinase pathways when ectopically expressed in BaF3 cells (*Online Supplementary Figure S2B*) for 8 representative mutants. As a control for this experiment, we used an autonomous BaF3 cell line obtained by transfection with an oncogenic mutant of M-RAS (Glu71Lys), which shows constitutive activation of the MAP kinase but not the

JAK/STAT pathway (*Online Supplementary Figure S2B*). Altogether, these results confirmed that the JAK1 mutations occurring spontaneously *in vitro* were JAK1/STAT5-activating mutations.

The F958V mutation confers resistance to ATP-competitive JAK inhibitors

JAK inhibitors represent a promising therapy for JAK mutation-positive neoplasms. We used our JAK1 mutation-positive autonomous cell lines to evaluate sensitivity of different JAK1 mutants to treatment with CMP6 inhibitor (JAK inhibitor I) by assessing tyrosine phosphorylation of JAK1 and STAT5, one of the main JAK1 phospho-substrates. As shown in Figure 2A, treatment of BaF3 Aut(V658I) with CMP6 completely blocked phosphorylation of JAK1 and STAT5, while the same treatment did not affect either JAK1 or STAT5 phosphorylation in the BaF3 Aut(F958V) cells. Similar results were obtained with BaF3 stably transduced with the corresponding JAK1 mutants (Figure 2B), confirming that the F958V mutation confers resistance to CMP6. To visualize the Phe958 to Val mutation, we used the crystal structure of the JAK1 kinase domain with CMP6 inhibitor (Figure 2C). The Phe958 is in direct interaction with CMP6 through its main-chain atoms as previously reported in this model (Figure 2C, left panel). The replacement of the Phe958 by Val abolishes this interaction (Figure 2C, right panel).

We further measured proliferation of BaF3 Aut(V658F) and Aut(F958V) in the presence of different concentrations of CMP6 or INCB018424. The BaF3 Aut(F958V) cells were more resistant to the effect of these inhibitors when compared to the Aut(V658F) cells, with a 10-15 times increase in the IC50 (Figure 2D and E). In contrast, no difference was observed in the sensitivity of these cell lines to cytosine arabinoside (ARA-C), a standard chemotherapeutic drug which targets different pathways (*data not shown*). Conversely, JAK inhibitors did not affect MAP kinase-dependent proliferation of control autonomous cells transfected with an activated mutant of M-RAS. In line with these results, treatment of Aut(V658) cells with INCB018424 blocked constitutive phosphorylation of JAK1 and STAT5, while their phosphorylation was preserved in Aut(F958V) cells even after treatment with 10 μM of inhibitor (Figure 2F). Altogether, these results show that the F958V mutation confers resistance to ATP-competitive JAK inhibitors.

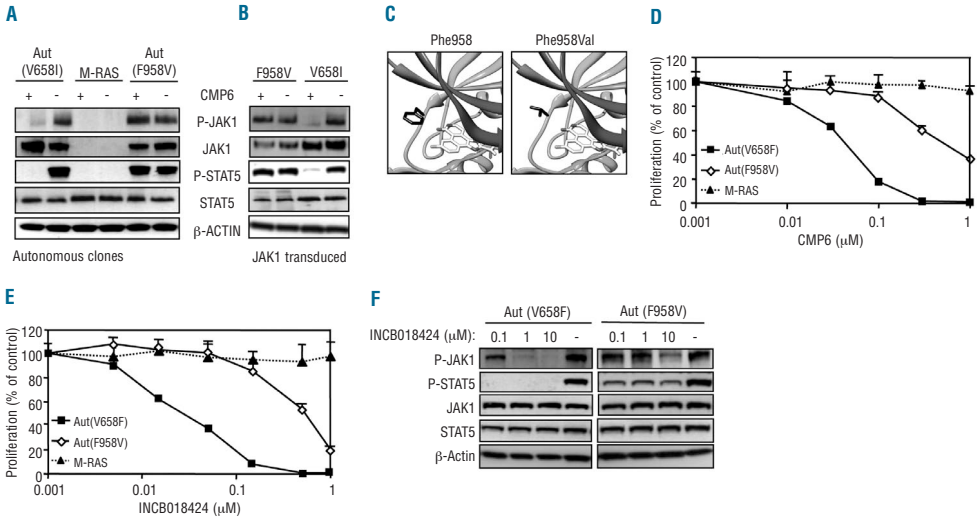


Figure 2. The F958V mutation confers resistance to ATP-competitive JAK inhibitors. (A) 10^6 BaF3 Aut(V658I), Aut(F958V) and BaF3 cells stably transduced with M-RAS or (B) JAK1 V658I and F958V mutants were treated for 30 min with 500 nM CMP6 inhibitor or with 0.1 % DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-p1034/35 JAK1 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments. (C) Magnification of the hinge region and ATP-binding pocket in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 (PDB 3EYH) in ribbon representation[21] showing the side chains of the Phe and Val residue at positions 958 for the wild-type JAK1 (left panel) and the F958V mutant (right panel), respectively. (D and E) 10,000 BaF3 Aut(V658F), Aut(F958V) cells or BaF3 cells stably transduced with activated M-RAS as a control, were seeded in 96-well plates and treated with increasing concentration of (D) CMP6 or (E) INCB018424 JAK inhibitor. After 48 h, tritiated thymidine was added to the cells for 4 h and thymidine incorporation was measured. Results are mean ± SD of triplicate cultures. Similar results were obtained in at least 3 independent experiments with 2 different autonomous clones for each JAK1 mutation. (F) 10^6 BaF3 Aut(V658I), Aut(F958V) were treated for 30 min with 100 nM, 1 μM or 10 μM INCB018424 inhibitor or with DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-pY1034/35 JAK1 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.

Mutations targeting JAK1 Phe958 and Pro960 confer JAK inhibitor resistance

To assess the sensitivity of other JAK1 mutation-positive cell lines to JAK inhibition, we performed a proliferation assay with increasing doses of INCB018424. The concentration of INCB018424 necessary to inhibit 50% of cell proliferation is represented in the *Online Supplementary Figure S3A*. Out of the 25 mutants, 6 showed a significant reduction in their sensitivity to INCB018424 (*Online Supplementary Figure S3A*). The other 19 mutants had the same sensitivity as the V658F (*data not shown*). All the mutants affecting Phe958 conferred BaF3 resistance to the inhibitor, as already shown for the F958V, with a 5 to 15-fold increase in the IC_{50} compared to V658F mutation-positive cells. The mutations of Pro960 to Thr or Ser, affecting a residue close to the Phe958, also reduced sensitivity to INCB018424 with an approximately 2 to 4-fold increase in IC_{50}. Similar results were observed when we tested CMP6 and other ATP-competitive inhibitors (*data not shown*). In line with these observations, all F958- and P960-targeting JAK1 mutations showed reduced inhibition of STAT5 phosphorylation in response to CMP6, while all the other mutations had similar IC_{50} to that of the parental BaF3 cells growing in the presence of IL-3 (*data not shown*).

One of the main mechanisms of secondary resistance in patients treated with tyrosine kinase inhibitors is acquisition of new inhibitor-resistant mutations. The JAK1 V658F mutation is constitutively active[26] and has been described in ALL and AML patients.[10,11] This makes it a good candidate for JAK inhibitor therapy. We decided to investigate whether the acquisition of a secondary JAK inhibitor resistant mutation in the kinase domain would make JAK1 V658F insensitive to JAK inhibition. We, therefore, transduced parental BaF3 cells with the V658F (JAK inhibitor sensitive), F958C (JAK inhibitor resistant) and the V658F/F958C double JAK1 mutants and selected these cells for autonomous proliferation. We then measured the proliferation of these cells with increasing concentrations of INCB018424 and calculated the IC50 (*Online Supplementary Figure S3B*). As expected, BaF3 cells expressing the F958C mutant were less sensitive than BaF3 V658F, while the level of JAK1 mutant expression was identical (*Online Supplementary Figure S3C*). Importantly, cells expressing the double mutants V658F/F958C showed identical sensitivity to the inhibitor as cells expressing F958C mutant alone (*Online Supplementary Figure S3B*). These results show that acquisition of a JAK inhibitor resistant mutation such as the F958C in cells dependent on activated JAK1 for growth is a potential mechanism of secondary resistance to JAK inhibitor therapy.

Long-term culture of F958V-positive BaF3 cells with JAK inhibitor leads to increased expression of F958V JAK1 that correlates with increased resistance to JAK inhibitor

We demonstrated that the F958V JAK1 mutation makes BaF3 cells resistant to JAK inhibitors. Therefore, proliferation of the F958V-positive cells in the presence of the JAK inhibitor CMP6 is not completely abolished and allows further expansion and proliferation of these cells in the presence of the JAK inhibitor CMP6 for days or even weeks (*Online Supplementary Figure S4A*). We tested the sensitivity of the F958V-positive cells to CMP6 before and after long-term culture in the presence of this inhibitor. Cells cultured with CMP6 for two weeks were more resistant to CMP6 than F958V-positive cells which had never been exposed to this inhibitor before (*Online Supplementary Figure S4B*). The JAK1 cDNA from several clones from this long-term culture have been entirely sequenced to rule out secondary acquired mutations. No secondary mutations have been detected in these cell lines. However, this increase in intrinsic resistance correlated with increased expression of JAK1 protein and RNA levels (*Online Supplementary Figure S4C and D*). Similar results were observed using F958C-positive cells (*Online Supplementary Figure S4D*). Altogether these results show that treatment of cells expressing JAK inhibitor-resistant mutants with JAK inhibitor allows an increase in the intrinsic resistance of these cells by increasing the level of mutated JAK1.

JAK2 Y931C, the homologous mutation of JAK1 F958C, confers resistance to JAK inhibitors

Because of its central role in the pathophysiology of MPNs, the JAK2 V617F is an attractive therapeutic target.[8] Currently several JAK2 inhibitors are under development or/and undergoing clinical trials for MPNs.[27] Identification of JAK inhibitor-resistant mutations in JAK2 is, therefore, of great importance. We, therefore, introduced the Y931C, a homologous mutation of JAK1 F958C, into WT or V617F JAK2 protein and selected these cells for autonomous proliferation. All three JAK2 mutants, but not JAK2 WT or control BaF3 cells, were able to proliferate in the absence of IL-3 (Figure 3A). The ability to proliferate autonomously was associated with constitutive JAK2 and STAT5 activation in these cells (Figure 3B). Proliferation of the autonomous cells was measured in the presence of increasing concentrations of INCB018424 or CMP6 to calculate the IC_{50} (Figure 3C and D). The JAK2 Y931C mutation dramatically reduced sensitivity of BaF3 cells to INCB018424 compared to JAK2 V617F-positive BaF3 cells (Figure 3C). Moreover, cells expressing the V617F/Y931C double JAK2 mutant showed identical sensitivity to the inhibitors as cells expressing Y931C mutant alone, with a 20 to 50-fold increase in the IC50 (INCB018424 and CMP6, respectively) compared to V658F mutation-positive cells. Similar results were observed when we tested other ATP-competitive inhibitors (*data not shown*). The resistance of the JAK2 Y931C positive cells to inhibitor was associated with persistent JAK2-phosphorylation in the presence of JAK inhibitor (Figure 3E). These results show that acquisition of a JAK inhibitor-resistant mutation such as the Y931C in cells dependent on activated JAK2 V617F for growth is a potential mechanism of secondary resistance to JAK inhibitor therapy in MPN patients.

Discussion

The acquisition of growth signal self-sufficiency is one of the hallmarks of cancer.[28] In this paper, we show that a broad spectrum of spontaneous mutations in one of the endogenous JAK1 alleles is the main mechanism that confers to BaF3 cells cytokine-independent growth capability *in vitro* and full tumorigenicity *in vivo* (*Online Supplementary Figure S5*), secondary to the upregulation of the JAK1 transcript. We use this *in vitro* model of spontaneous tu-

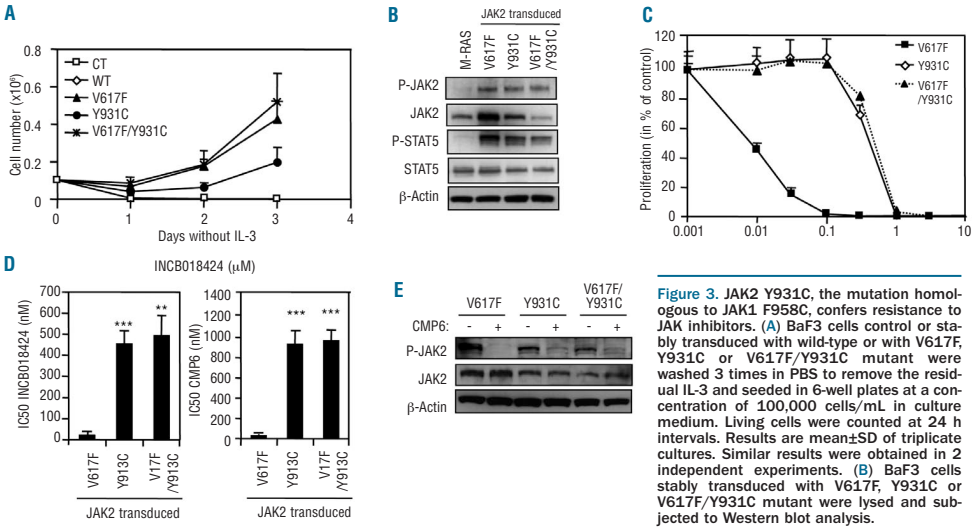


Figure 3. JAK2 Y931C, the mutation homologous to JAK1 F958C, confers resistance to JAK inhibitors. (A) BaF3 cells control or stably transduced with wild-type or with V617F, Y931C or V617F/Y931C mutant were washed 3 times in PBS to remove the residual IL-3 and seeded in 6-well plates at a concentration of 100,000 cells/mL in culture medium. Living cells were counted at 24 h intervals. Results are mean±SD of triplicate cultures. Similar results were obtained in 2 independent experiments. (B) BaF3 cells stably transduced with V617F, Y931C or V617F/Y931C mutant were lysed and subjected to Western blot analysis. Phosphorylation of JAK2 and STAT5 was detected using specific anti-pY1007/8 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK2, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments. (C) 10,000 BaF3 cells stably transduced with V617F, Y931C or V617F/Y931C JAK2 mutants were seeded in 96-well plates and treated with increasing concentration of INCB018424 JAK inhibitor. After 48 h, tritiated thymidine was added to the cells for 4 h and thymidine incorporation was measured. Results are mean ± SD of triplicate cultures. Similar results were obtained in at least 3 independent experiments. (D) IC50 for INCB08424 and CMP6 inhibitor calculated based on dose-dependent proliferation curves of BaF3 cells stably transduced by V617F, Y931C and V617F/Y931C double JAK2 mutant. Results are mean ± SEM of at least 3 independent experiments (P<0.01; ***P<0.001). (E) 10^6 BaF3 cells stably transduced with JAK2 V617F, Y931C and V617F/Y931C double JAK2 mutant were treated for 30 min with 300 nM CMP6 inhibitor or with DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK2 was detected using specific anti-pY1007/8 JAK2. Membranes were re-probed with anti-JAK2 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.**

morigenesis to generate a library of cell lines with 25 different *de novo* JAK1-activating mutations, 6 of which are targeting two residues, Phe958 and Pro960, located in the hinge region at the periphery of the ATP-binding pocket. The important property of these mutations targeting Phe958 and Pro960 is that they not only make the kinase constitutively active, but also give increased resistance to ATP-competitive inhibitors, including INCB018424, which is currently used in clinical trials for JAK2 V617F-positive MPNs.[12]

The major drawback of tyrosine kinase inhibitor therapy is the development of secondary resistance caused by the acquisition of new mutations, as best exemplified by imatinib-resistant mutations in BCR-ABL positive CML.[13] Multiple BCR-ABL kinase domain mutations conferring imatinib-resistance have been described including the T315I mutation, which targets the gate-keeper Thr315 in ABL kinase. This gatekeeper Thr residue is not conserved in the JAK kinase family, but the Phe958 residue of JAK1 is the homologous residue of Phe317 in ABL kinase, which is close to the gatekeeper residue and was also found to be mutated in imatinib-resistant BCR-ABL positive patients[13,29,30] (*Online Supplementary Figure S6A and S6B*). Along the same lines, in non-small cell lung cancer (NSCLC), acquired resistance to Gefitinib/Erlotinib kinase inhibitors has also been associated with a secondary mutation of the gatekeeper Thr790 residue of the epidermal growth factor receptor (EGFR). The T790M mutation has been shown to activate the wild-type EGFR, acting both as a drug-resistance and as an activating mutation, similar to JAK mutations reported here.[31]

The gatekeeper Thr residue in ABL and EGFR is located at the periphery of the nucleotide-binding site and directly interacts with imatinib, so that mutation into more bulky amino acids such as Met and Ile sterically hinders the binding of imatinib and other ATP-competitive inhibitors. However, at least for the EGFR, increased affinity for ATP appears to be the primary mechanism by which the T790M mutation confers drug resistance, and might also explain its activating property.[32] Based on the crystal structure of the JAK1 kinase domain (PDB 3EYH),[21] replacement of Phe at position 958 by the less voluminous Val, Cys, Ser or Leu residues is predicted to change the orientation of the side chain to the opposite direction (Figure 2B). This rules out the possibility that resistance to inhibitors results from a steric hindrance as proposed for the Thr315 mutations of ABL.[13,33] However, the crystal structures of JAK1 and JAK2 also show that Phe958 or Tyr931, respectively, are in direct contact with the CMP6 inhibitor.[21] The replacement of Phe958 by Val/Cys/Ser/Leu abolishes this

interaction and should decrease the affinity for this compound (Figure 2C). Further structural data are necessary to determine whether a critical interaction also takes place between Phe958 and INCB018424 or other inhibitors used in this study, but the observation that these JAK mutations confer resistance to different ATP-competitive inhibitors and that they are activating mutations is more in line with a mechanism of increased affinity for ATP, as shown for the T970M EGFR mutation.[32] The homologous residues to JAK1 Phe958 are either Phe or Tyr in other kinases such as ABL, SRC, PDGFR, KIT and JAK2, suggesting that an aromatic side chain at this position is required for proper regulation of the kinase activity. The effect of mutations affecting Phe958 in JAK1 and Tyr931 in JAK2, combined with the F317L/I mutations in drug-resistant BCR-ABL patients supports the notion that this residue plays a critical functional role for the ATP access into the ATP-binding pocket.

The clinical relevance of our *in vitro* model is highlighted by the fact that 5 of the 25 activating mutations (S646F, Y652H, V658F, R724H, L783F) have been previously reported in human patients. The 6 drug-resistant mutations have not yet been described in patients, but our observation that Tyr931 mutation confers resistance of JAK2 V617F to several JAK inhibitors, including INCB018424, suggest that these mutations might contribute to the development of drug-resistant clones during treatment of JAK2 V617F-positive MPNs with such inhibitors.

It has been shown in imatinib-resistant CML that drug resistance conferred by mutations does not necessarily correlate with proliferative advantage and increased kinase activity.[34] Other, non-activating mutations or drug-resistance mechanisms, might be acquired by tumor cells. However, as proposed for the T790M mutation in the EGFR,[31] the significant gain-of-function property conferred by the mutations that we describe here may favor their initial presence before drug selection, and rapid selection during tyrosine kinase inhibitor-based therapy. These JAK mutants should, therefore, be considered to be an invaluable tool to evaluate the activity of novel, potentially more potent, ATP-competitive JAK inhibitors for MPNs and other neoplastic processes associated with JAK kinase mutations.

Authorship and Disclosures

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Annex

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