Original Article

The JAK2V617F Point Mutation Increases the Osteoclast Forming Ability of Monocytes in Patients with Chronic Myeloproliferative Neoplasms and Makes their Osteoclasts more Susceptible to JAK2 Inhibition

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Abstract. *JAK2V617F* is a gain of function point mutation that occurs in Myeloproliferative Neoplasm (MPN) patients and deranges their hemopoiesis at cellular level. We speculate that hyperfunctioning JAK2 can modify osteoclast (OCL) homeostasis in MPN patients. We studied 18 newly diagnosed MPN patients and four age-matched normal donors (ND). Osteoclast forming assays started from selected monocytes also and under titrated concentrations of the JAK2 Inhibitor AG-490 (Tyrphostin). Genomic DNA was extracted from the formed osteoclasts, and the *JAK2V617F/JAK2WT* genomic DNA ratio was calculated. OCLs formed from monocytes derived from heterozygous (Het) for the *JAK2V617F* mutation MPN patients, were three times more compared to those from *JAK2* wild type (WT) MPN patients (p=0,05) and from ND as well (p=0,03). The ratio of *JAK2V617F/JAK2WT* genomic DNA was increased in OCLs compared to the input monocyte cells showing a survival advantage of the mutated clone. In comparison to ND and JAK2 WT MPN patients, OCLs from patients *JAK2V617F* (Het) were more susceptible to *JAK2* inhibition. These alterations in osteoclast homeostasis, attributed to mutated *JAK2*, can deregulate the hemopoietic stem cell niche in MPN patients.

Keywords: JAK2V617F, Osteoclast, Myeloproliferative neoplasm.

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Introduction. Osteoclasts (OCLs) are bone resorting cells of hemopoietic origin. The balanced function between osteoclasts and osteoblasts determines bone homeostasis. Osteoblasts are considered important cells for the hemopoietic stem cell (HSC) niche formation and regulation,[1] while osteoclasts are considered dispensable cells in this setting. Their role is to mobilise hemopoietic progenitor cells towards the peripheral blood especially under stress conditions.[2-6]

JAK2 is an intracellular kinase, a cellular hub, that acts as a second mediator of intracellular signaling from a variety of cytokine and hormone receptors.[7,8] Pharmacological inhibition of the *JAK2* kinase reduces osteoclast bone resorting activity,[9-12] suggesting that the intact *JAK2* function is essential for normal osteoclast

function.

The *JAK2V617F* is a gain of function point mutation that often occurs in patients with myeloproliferative neoplasms (MPN) and deranges basic cellular functions of their hemopoietic cells.[13,14] *JAK2V617F* arises in an early myeloid progenitor cell, cells ancestor to OCLs. Except intrinsic stem cell defects, HSC niche deregulation in *JAK2V617F* mutated mice models of MPN contributes equally to MPN disease evolution.[1,15]

Therefore we speculate that genetically modified *JAK2*, as it occurs in MPN patients, can slightly modify osteoclast homeostasis in a way that deregulates hematopoietic stem cell niche homeostasis.

Patient's Samples and Materials. Approximately, 60 ml of peripheral blood was drawn, after informed contest form was signed, from 18 newly diagnosed MPN patients (14 essential thrombocythemia, two primary myelofibrosis, two polycythemia vera diagnosed according to 2008 WHO criteria) and from 4 age-matched normal donors (ND). Positive selection of CD14+ monocyte cells was performed by using immunomagnetic beads separation technique (MiltenyiBiotec). The selective JAK2 inhibitor, TyrphostinAG-490, was provided by Sigma Diagnostics and was dissolved in DMSO. This study was approved by the local ethics committee and conducted according to the principles of the declaration of the Helsinki.

JAK2V617F mutation detection in our patient's cohort. Genomic DNA was extracted from whole blood (QIAGEN), and 25 ng of gDNA were subjected to Allele-specific PCR (ASO-PCR) for the detection of the *JAK2V617F* mutation according to the protocol described by Baxter et al., slightly modified. Restriction Length Polymorphism method (RFLP) by using the BsaXI restriction enzyme, was performed in all positive for the *JAK2V617F* mutation samples, to determine its presence in heterozygous or homozygous status.

Osteoclast (OCL) forming assays from monocyte cells derived from MPN patients. $2x10^5$ positively selected monocyte cells, after checked for purity by flow cytometry, were suspended in a MEM medium + 10% Fetal Calf Serum, supplemented with optimum concentrations of osteclastogenic cytokines (RANKL 25 ng/ml and M-CSF 50 ng/ml) and then they were plated in a 48-flat bottom well plate. The half medium exchange was performed twice weekly, and the number of formed OCLs was scored under inverted microscope by using TRAP assays (Sigma Diagnostics) after staying 14 days in culture.

Quantitative Real-Time PCR (RT-PCR) for JAK2V617F/JAK2WT genomic DNA ratio measurement. Genomic DNA was extracted (QIAGEN) from both the input for OCL forming assays monocyte cells as well as from the OCLs that they were finally formed from them. *JAK2V617F/JAK2WT* genomic DNA ratio was calculated by using the Taqman based JAK2MutaQuant Kit (IPSOGEN). Briefly, a pair of FAM-tracked hybridisation probes was used in two different reaction tubes, one of them specific for the G1849T *JAK2* mutation and the other specific for the wild type sequence, together with a *JAK2*gDNA specific pair of primers. Appropriate serial dilutions of known numbers of mutated and wild type *JAK2* transcripts were used in order to set the calibration plot. All assays were performed on an ABI Prism7500 analyser (Applied-Biosystems).

OCL forming assays in the presence of the JAK2 inhibitorAG-490(Tyrphostin). Initially, titration of the inhibitory dose of AG-490 on OCL formation assays was performed. Selected monocytes from three normal donors started OCL forming assays in the presence of serial dilutions of AG-490 dissolved in DMSO; range 0.01-100 μM. The 50% Inhibitory concentration (IC50) was determined at 20μM (**Figure 2A**). Then OCL forming assays were started from MPN patient's monocytes as previously described, and concurrently OCL forming assays in the presence of 2μM and 20μM of the JAK2 inhibitor AG-490 (Tyrphostin) dissolved in DMSO were also started.
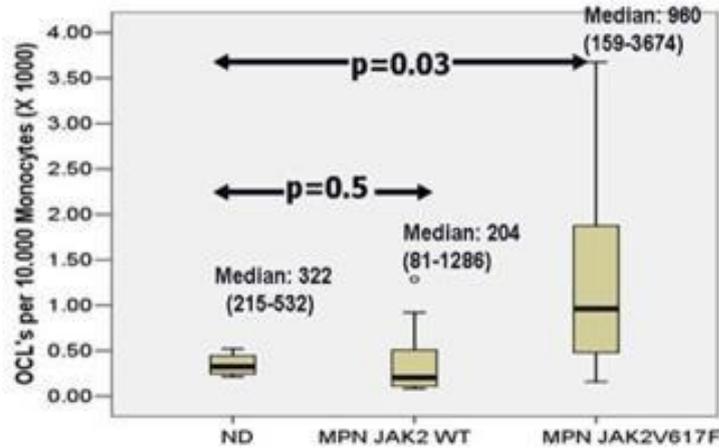
CTXβ in serum measurement by ELISA and Colony Forming Assays from CD34 selected cells from the peripheral blood. CTXβ levels in whole blood serum were measured by ELISA (β-crossLaps/serum kit, Roche Diagnostics). Data are expressed as ng/ml.
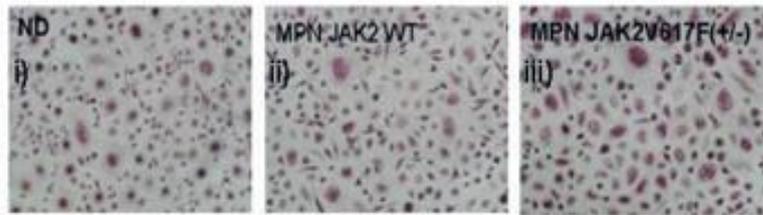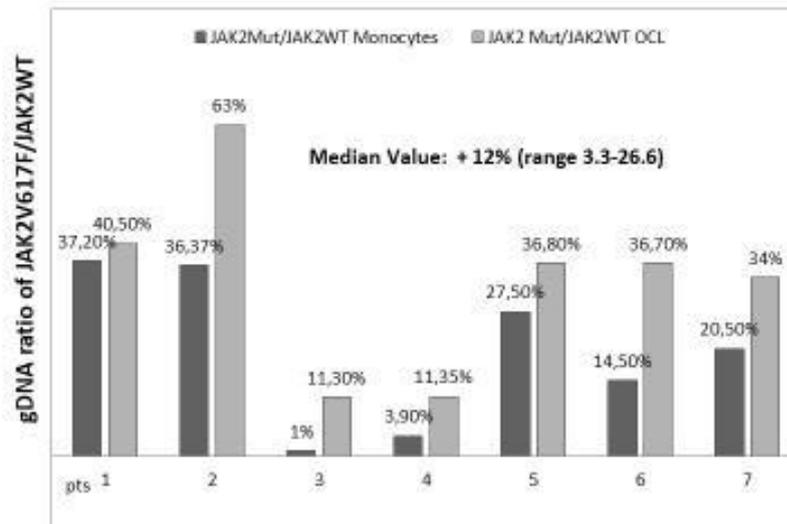
Figure 1. Osteoclast Forming assays and ratio of JAKWT/JAK2V617FgDNA copies in monocytes and in the osteoclasts derived from them. A. An example of TRAP + osteoclasts derived from selected monocytes after staying in OCL forming assays for 14 days i) normal donors, ii) JAK2 WT MPN patients, iii) JAK2V617F (Het) MPN patients. **B.** Osteoclasts formed from the same number of monocyte cells under optimum osteoclastogenic conditions (M-CSF and RANKL) are significantly more for JAK2V617F (Het) MPN patients compared to normal donors (Mann-Whitney, p=0,03). **C.** Ratio of JAKWT/JAK2V617FgDNA copies were measured by Taq-man based Real-Time PCR assays in the input monocyte cells and in the osteoclasts that were finally derived from them. The osteoclasts overgrowth noted in JAK2V617F (Het) MPN patients is partly due to the overgrowth of the mutated clone (median expansion: 12%, range 3,3-26,6%).

Statistical analysis. All values represent median values (min-max). Comparisons between groups were performed by using the Mann-Whitney for unpaired and the Wilconxon Rank test for paired samples. Statistical significance was considered at the level of p<0.05 value. The SPSS-13 statistical package was used for statistical analysis.

Results.

JAK2 mutation status detection. Nine MPN patients out of eighteen were found positive for the *JAK2V617F* point mutation (2 PV patients, 1 IMF patient and 6 ET patients) while nine MPN patients (8 ET patients and 1 IMF patient), and the four ND were *JAK2V617F* negative. Apparently, two JAK2 WT patients were tested positive for the CALR mutation and none positive for cMPL mutation. We then subjected the samples that were

positive for *JAK2V617F* mutation to RFLP assays by using the BsaXI restriction enzyme, to determine their ploidy status. All out of nine patients tested were heterozygous for the *JAK2V617F* point mutation.

The presence of the JAK2V617F point mutation enhances in-vitro OCL forming ability. OCLs were formed from monocyte cells derived from ND, and *JAK2* mutated or unmutated MPN patients (an example is given in **Figure 1A**). OCLs derived from heterozygous for the *JAK2V617F* mutation (Het) MPN patients, were significantly more compared to those from the *JAK2* wild type (WT) MPN patients and ND as well; Median OCLs per 10.000 monocytes, (min- max): *JAK2V617F* (Het) MPN: 960 (159-3674), *JAK2* WT MPN: 204 (81-1286) and p=0.03 Mann-Whitney test (**Figure 1B**). Considering ND the Median (min-max) OCLs per 10.000 monocytes were: 322 (215-522) and p=0.05 when compared to *JAK2V617F* (Het) MPN patients (**Figure 1B**).

OCLs derived from JAK2V617F(Het) monocytes are enriched for the mutated clone compared to the input monocyte cells reflecting an increased outgrowth of the JAK2 V617F mutated clone. We then performed quantitative Taqman based RT-PCR assays at genomic DNA level, in both the input monocyte cells and in the OCLs that were finally derived from them, aiming to clarify whether the increased OCL formation noted from *JAK2V617F* (Het) MPN monocytes reflects outgrowth of the wild type or the mutated clone. In all out of seven *JAK2V617F* (Het) MPN patients successfully tested, the ratio of *JAK2V617F/JAK2WT* genomic DNA was increased in the OCLs fraction compared to the input monocyte cells. The median enrichment of OCLs for the *JAK2V617F* mutated clone was 12% (range: 3.3%-26%) and p<0.01, Wilconxon Rank test (**Figure 1C**).

No evidence by CTXβ of increased OCL activity in JAK2 V617F patients. The ability of JAK2 (Het) for higher OCL formation ex vivo poses the question if these patients have in higher levels of osteoclast activity in vivo. Therefore we quantified in serum from our MPN patients the levels of CTXβ by ELISA but we cannot find any difference among groups [median Value (min-max) JAK2 (Het): 1,013(0,9-2,1 ng/ml) JAK2

WT: 1,014(0,78-2,1 ng/ml, p=0,69 Data not shown).

In Vitro Osteoclastogenesis from monocyte cells derived from MPN patients is dependent upon JAK2 function. In order to address the dependence of osteoclastogenesis from JAK2 kinase in mutated and unmutated cells we performed OCL forming assays under titrated inhibition of the JAK2 kinase by the selective JAK2 inhibitor AG-490. We determined IC50 in OCL forming assays performed from 3 ND monocytes at 20μM (**Figure 2A**). Afterwards, we performed OCL inhibition assays under titrated concentrations of AG490 at 2μM and 20μM in MPN patients. OCL inhibition is dose dependent in both groups of MPN patients (**Table 1**) but it has a significantly suppressing effect on OCL formation only in JAK2 (Het) MPN patients compared to ND. Of Note, at the higher inhibitory concentration of 20 μM OCL formation in samples from JAK2V617F (Het) patients was significantly reduced compared to ND (**Table 1**, p=0,02) and compared to *JAK2* WT MPN patients as well (p=0,05). There was no statistically significant difference between ND and *JAK2* WT MPN (p=0,28, **Figure 2B**).

Discussion. In this study, we demonstrated that cells carrying the *JAK2V617F* point mutation are more efficient for osteoclast formation compared to cells carrying the *JAK2* WT genotype. Monocytes derived from *JAK2V617*(Het) MPN patients can form 3 times more OCLs compared to the monocyte cells carrying the *JAK2* WT genotype from both MPN patients and ND. Because input monocyte cells from *JAK2V617*(Het) patients is a mixture of cell clones carrying both *JAK2V617F* and *JAK2 WT* DNA copies we performed real-time PCR at genome level to see whether the OCL overgrowth is due to the preferential expansion of either of the two clones. We demonstrated that the percentage of mutated *JAK2* DNA copy numbers in the OCL fraction was increased compared to the input monocyte cell fraction by a median value of 12%. In our culture system the concentrations of RANKL and MCSF is optimum for monocyte differentiation to osteoclasts, and therefore cells committed to the OCL lineage are attached to the bottom of the plate, and they will mature to OCL unless they do not survive. Hence, the enrichment of OCL cell fraction for V617F mutated clones
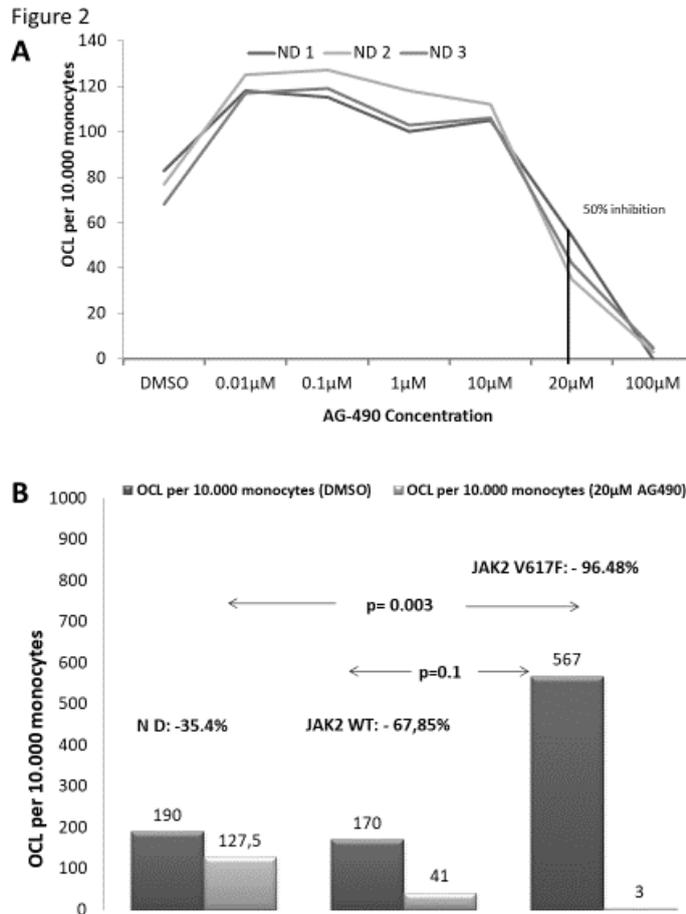
Figure 2. Inhibition of OCL forming assays with the JAK2 inhibitor AG-490 (Tyrphostin). A. Osteoclast forming assays were performed under titrated concentrations of the selective *JAK2* inhibitor AG-490 dissolved in DMSO. Monocyte cells from 3 ND were used in order to determine the 50% inhibitory concentration of AG-490. The 50% inhibitory concentration was 20μM. B. Osteoclast forming assays from selected monocyte cells derived from MPN patients under the concentration of 2μMand of 20μM of AG-490. Compared to ND osteoclast formation was almost completely abolished in *JAK2V617F* (Het) MPN patients and was equally reduced in ND and in *JAK2* WT MPN patients.

Table 1. Median OCL formed under titrated inhibition with the JAK2 inhibitor AG490.

	AG490 0μM	AG490 2μM	AG490 20μM	Median % of Reduction 0μMvs 2μM	Median % of Reduction 0μMvs 20μM
ND (Median, min-max)	201 156-232	190 156-428	127 79-214	0% 0-12	35,4% 29-50
JAK2 WT(Median, min-max)	204 81-1286	170 80-487	41 0-250	11% 1-81	84,4% 34-100
JAK2 V617F (Median, Min-max)	960 159-3674	567 70-2647	3 0-250	27% 11-55	97,4% 45-100

shows a proliferation/survival advantage of the mutated clone compared to the JAK2 WT clones.

We then tried to address if this ex vivo effect of JAK2V617F on OCL formation has a detectable in vivo effect. Therefore we assessed osteoclastogenic activity in the serum of our patients by measuring CTXβ levels finding no difference between both groups. These data suggest that the in vivo effect of JAK2V617F on OCL formation and finally on the bones is not so robust in order to be detected by CTXβ levels.

In order to provide further insight to the dependence of MPN osteoclastogenesis from the *JAK2* kinase function, we performed OCL forming assays under titrated pharmacological inhibition of the *JAK2* kinase with the selective *JAK2* inhibitor AG-490 (Tyrphostin). We noted that osteoclastogenesis from cells carrying the *JAK2V617F* point mutation is more susceptible to *JAK2* kinase inhibition than osteoclastogenesis from cells that are carrying the wild type *JAK2* kinase in a dose dependent manner. Our data

provide evidence for a therapeutic effect of JAK2 inhibitors in MPN patients through reversion of OCL deregulation.

Our data enforce a model of deregulated osteoclastogenesis in a disease characterised by the genetically modified JAK2 kinase. In this study, we found out that the acquisition of the *JAK2V617F* point mutation in hematopoietic cells capable of osteoclastogenesis increases their OCL forming ability, but these OCLs are also more vulnerable to *JAK2* kinase inhibition. This poses to us the challenge to extrapolate the underlying mechanism of increased osteoclastogenesis in JAK2V617F mutated cells and furthermore to perform OCL functional assays in a cohort of MPN patients enriched in patients homozygous for the JAK2 mutation. Targeting osteoclasts may be of value in MPN patients.

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