Research on the Influence of new oncogenic *CSF3R* mutations in Chronic Neutrophilic Leukemia

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In this research, we will be mainly focusing on the new genetic findings, such as granulocyte-colony stimulating factor receptor (CSF3R) mutations, especially a novel mutation site CSF3R-T618I, as well as with an unreported novel mutation site of CSF3R- H54A, and their influence on the molecular pathogenesis of the Chronic Neutrophilic Leukemia.

Part I. Case presentation: Chronic neutrophilic leukemia with overexpression EVI-1 gene and concurrent CSF3R and SETBP1 mutation

Background: Chronic neutrophilic leukemia (CNL) is a rare myeloproliferative neoplasm (MPN) characterized by sustained neutrophilia, splenomegaly, bone marrow granulocytic hyperplasia without evidence dysplasia and absence of the Philadelphia (Ph) chromosome. The case presented herein was characterized by marked and sustained neutrophilia, increased NAP score, absence of Ph chromosome, absence of bcr-abl fusion transcripts on RT-PCR, and exclusion of underlying disorders causing the leukemoid reaction. In the present case, on the first admission patient was no hepatosplenomegaly, but other laboratory findings satisfied the WHO diagnostic criteria. One year later of the first admission, the patient suffered from bleeding gums, splenomegaly, and lymphadenopathy due to disease progression. In our patient the mean value of peripheral blood cell count during the clinical course were as follows; leukocyte count 28.36 X 109/l (range 7.67-51.28 X109/l) with 88.5% mature neutrophils (range 78.8-98.5%), platelet count 33.3 X 109/l (range 23-94 X 109/l). Thrombocytopenia was observed in our patient at presentation and followed a progressive course without responding to treatment with hydroxyurea and IFN-α.

Materials and Methods: Peripheral Blood was collected from the CNL patient and PMNCs were collected using Ficoll Gradient density separation and grown in complete medium at 37°C in a humidified atmosphere composed of 5% CO2 and 95% air. Neutrophil function analysis, such as cell apoptosis, cell cycle and cell migration test were checked with LSR II Flow Cytometer. Neutrophils are cultured in RPMI 1640 medium with 10% FBS, at different time points, cells were collected and labelled with Annexin V and PI (BD Pharmingen), then test the apoptosis compared with the normal control group. Purified neutrophils were cultured in the transwell insert (1x106/well), and chemotactic peptide N-formyl-methionyl-leucyl-phenylalan (fMLP, 1uM, SIGMA) was added to the lower compartment. At each time point, we counted the number of the cells in the lower compartment. Oncogenic CSF3R mutation in a whole exon and intron by PCR-based DNA Pyrosequencing method on the CD34+, CD15+ cell fraction was sorted using a BD FACSAria III (BD Biosciences) flow cytometer.

Result: In patient's CBC has 93.9% of the cells were mature neutrophils. Flow cytometric analysis of cell apoptosis revealed that our patient's neutrophil still alive after 96 hours, but we found very few alive neutrophils on the normal control group after 72 hours. Flow cytometric analysis of the cell cycle revealed as a normal. Therein, all cells were terminally differentiated and there is no cell division. The extent of migration activity by fMLP was lower in the patient neutrophils compared to the normal control cells with fMLP, but higher than to normal negative control /without fMLP/. An interesting observation, EVI-1 gene-positive was found by a qualitative screening test. Then we checked the expression level of EVI-1 gene by cell sorting CD 34+, CD 15+ positive cell fraction and bone marrow sample by quantitative RT-PCR screening test 3 times during the clinical course. Interestingly, those tests results were similar, especially on the bone marrow sample and CD 34+ cell fraction detected overexpression EVI-1 gene, but on the CD15+ cell fraction EVI-1 gene was also positive, but the expression was low. The patient's result was positively detected the membrane-proximal mutation p.Thr618Ile on the exon 14 of CSF3R on both CD15+ and CD34+ cell fraction, in addition, a novel mutation site revealed on the exon 4 of CSF3R heterozygous mutation p.His54Asp. We also evaluated somatic SETBP1 mutational alteration analysis by PCR amplification followed by Sanger sequencing for codons between 706-917 in exon 3. The experiment result is indicated that the patient's somatic heterozygous SETBP1 mutation was encoding at p.Asp868Asn, c.2602C>A.

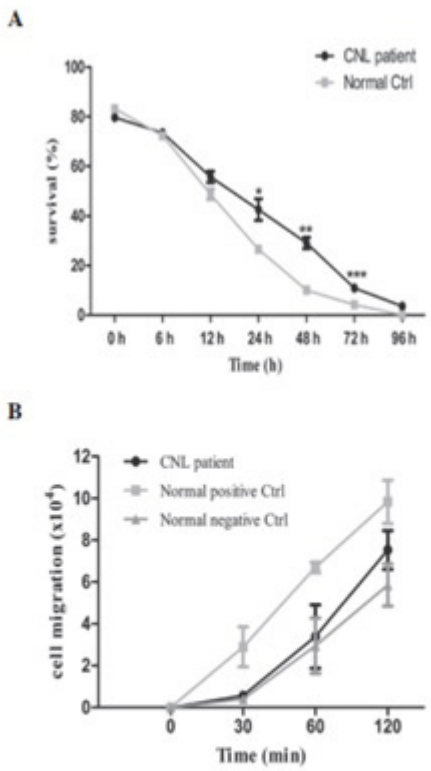


Figure 1. Cell survival curve and cell migration assay. (A) Cell survival data obtained at different time points indicating that the patient's neutrophils survive significantly longer than those of the normal control group. *P=0.210 vs. control; **P=0.0012 vs. control; and ***P=0.0010 vs. control. (B) Extent of cell migration, as determined by N-formyl-methionyl-leucyl-phenylalanine (fMLP) treatment. Migration of the patient neutrophils was low compared with the normal positive Ctrl cells (incubated with fMLP), but high compared with the normal negative Ctrl cells (incubated without fMLP). CNL, chronic neutrophilic leukemia; ctrl, control.

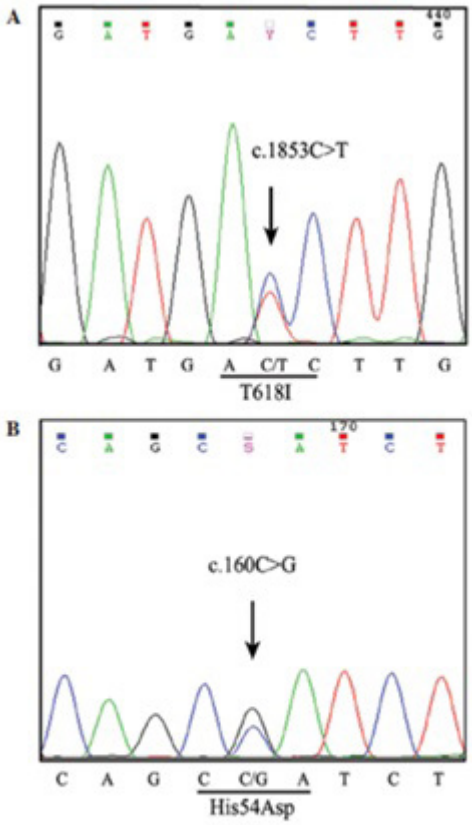


Figure 3. Detection of the T618I and His54Asp point mutations in colony stimulating factor 3 receptor (*CSF3R*). (A) Sequencing of exon 14 of *CSF3R* detected a base-pair mutation (C to T) at nucleotide 1853. (B) Sequencing of exon 4 of *CSF3R* detected a base-pair mutation (C to G) at nucleotide 160.

Table I. Qualitative and quantitative EVI-1 screening tests.

Test	Bone marrow sample	EVI-1 expression
Qualitative screening of 36 leukemic fusion genes		
September 13, 2012	Total	Positive
Quantitative screening of EVI-1 expression		
September 25, 2012	Total	499.87[a] (overexpression)
November 5, 2012	$CD34^+$ cell fraction	214.82[a] (overexpression)
November 5, 2012	$CD15^+$ cell fraction	34.21[a] (low expression)
May 15, 2012	Total	151.27[a] (overexpression)

[a]$2^{-\Delta\Delta Ct}$ values. When $2^{-\Delta\Delta Ct} \leq 8$, EVI-1 expression is negative; when $8 \leq 2^{-\Delta\Delta Ct} \leq 32$, EVI-1 gene expression is low; when $2^{-\Delta\Delta Ct} \geq 32$, EVI-1 is over-expressed. EVI-1, ectopic viral integration site-1, also known as *MECOM*; Ct, cycle threshold; CD, cluster of differentiation.

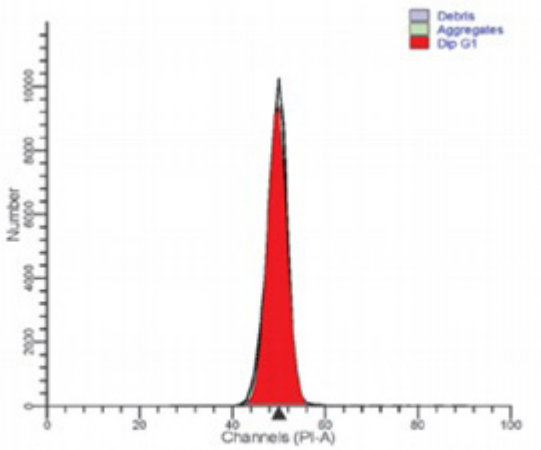


Figure 2. Cell cycle analysis determined by flow cytometry. All cells were terminally differentiated and there was no cell division.

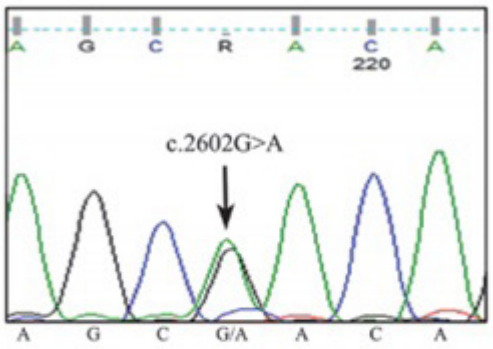


Figure 4. Detection of the p.Asp868Asn point mutation in the SET binding protein 1 gene.

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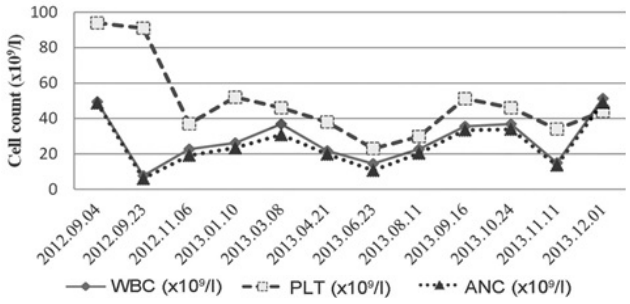


Figure 5. Cell count recorded throughout the disease course. WBC, white blood cells; PLT, platelets; ANC, absolute neutrophil count.

Conclusion: This is a case of a 67-years-old man presenting with sustained neutrophilia, persistent thrombocytopenia, absent BCR-ABL1 transcripts and JAK2 V617F mutation, in addition, an interestingly overexpressed EVI-1 gene and a new concurrent mutation of CSF3R and SETBP1, as well as with an unreported novel mutation site of CSF3R-H54A, ultimately diagnosed as CNL. Our next studies are needed to elucidate the mechanism of the mutation sites on the molecular pathogenesis of the CNL.

Part II. Study on the mutation sites of the CSF3R, which were responsible for the clonal advantage of CNL

Objective: To determine CSF3R-FL, CSF3R-T618I, CSF3R- H54A mutations have some significance on the molecular pathogenesis of CNL

Materials/Methods:

1. Plasmid construction. Plasmids were constructed by PCR amplification of the insert, restriction digestion and ligation using standard molecular biology methods, briefly: the host vectors pLV-EF1α-EYFP-N, pLP-2, pVSV-G, pLP-1 gag pol were purified with Omegabio-tek maxi prep kit and digested by restriction enzymes (ECO RI, NOTI). The linearized vectors were purified from agarose gel using an AxyPrep TM DNA Gel Extraction Kit and the concentration of the samples was estimated on an agarose gel stained with ethidium bromide. The inserts (CSF3R-FL, CSF3R-T618I, CSF3R- H54A) were generated by PCR, the sequences of the primer-pairs used and the conditions of the PCR reactions.

The amplified DNA fragments were purified from agarose gels using AxyPrep TM DNA Gel Extraction kits and digested by ECO RI, NOTI was used to linearize the acceptor vector. Enzymatic reactions in the case of the i) insert: 100–3000 ng purified PCR product was digested by an appropriate amount of enzyme and 4 μl of 10x reaction buffer in 40 μl of final volume for overnight at the appropriate temperature; ii) vector: 2000–4000 ng plasmid DNA was digested by an appropriate amount of enzyme and 2 μl of 10x reaction buffer in 20 μl of final volume for 4 hours at the appropriate temperature. When necessary the digested fragments were purified again and the concentrations of the inserts were estimated on agarose gels, as described above. A 1:3 vector: insert molar ratio was used for the ligation reactions. Chemically competent DH5α Escherichia coli bacteria were transformed with the products of the ligation reactions and were grown on Luria Bertrani (LB) agar plates containing the required antibiotic, such as ampicillin (Sigma). A day later single colonies were picked from the plate, inoculated into and grown overnight in LB medium containing with ampicillin. Plasmids were purified from the overnight cultures as above and tested by restriction mapping for the presence of the insert. Selected clones were sequenced by Sanger sequencing.

2. Lentiviral packaging system we used 3 main components, such as the lentiviral expression vector (Plasmid DNA of CSF3R-FL, CSF3R-T618I), the lentiviral packaging plasmids (pLP-1, pLP-2 plasmids that encode for gag, pol, and rev from the HIV or FIV genome and pVSV-G), 293TNN producer cells. We seed 1X105 293TNN cells per 10cm2 culture plate in 2-3 ml of culture medium containing DMEM medium supplemented with 4 mM L-glutamine, 4.5 g/l glucose, and fetal bovine serum (10%) without antibiotics. Grow for 18-24 hours at 37 °C with 5% CO2 so that the cell density reaches ~60 - 80% confluency at the time of transfection. We used a GFP as a positive control, to confirm transfection experiment was successful. Then we collected the cell culture supernatant, which is containing infectious pseudo viral particles.

3. Transduction of Pseudotyped Viral Particles into the primary cell of mouse. In the final step, we have used the Mouse Colony Forming Unit Assay using MethoCultTM to assess the effects of CNL-associated oncogenes on the morphology and number of primary murine cells derived bone marrow. For this purpose, cells are transduced with either control, which is without viral construct or a construct expressing the oncogene of interest (CSF3R-FL, CSF3R-T618I).

Results:

1. On the Plasmid construction step we successfully extracted and purified of recombinant plasmids of CSF3R-FL and CSF3R-T618I cloning, but we still did an experiment to obtain the recombinant plasmid CSF3R- H54A cloning.
2. After 24 hours of transfection 293TNN Cells with Packaging Plasmids and the Expression Construct, cells we visualized with green fluorescence protein under the fluorescence microscope.
3. The both two of CSF3R cloning were capable of transforming murine colony-forming cells. After transforming, CFU-GM colonies were counted manually by light microscopy seven days after plating. We found that the membrane-proximal mutation (T618I) transformed CFU-GM colonies number was more than the full-length non-mutants (CSF3R-FL), which indicates that T618 mutation of CSF3R conferred the clonal advantage of CNL leukemia cells.

Discussion:

Previously, two types of CSF3R mutations were found, the majority being membrane proximal mutations CSF3R-T618I (n=12) and CSF3R-T615A (n=2), either in isolation (n=9) or as a compound mutation with a variety of frameshift or nonsense mutations that truncate the cytoplasmic tail (truncation mutations) [6]. Eight of nine cases (89%) classified as CNL had CSF3R mutations compared to 44.4% (eight of eighteen) classified as either aCML or

ambiguously as “aCML favoured over CNL”. Pardanani et al. concluded that CSF3R-T618I is a highly sensitive and specific molecular marker for CNL and should be incorporated into current diagnostic criteria. In our case, the membrane proximal mutation CSF3R-T618I was confirmed and also unreported a novel mutation site H54A of CSF3R was identified.

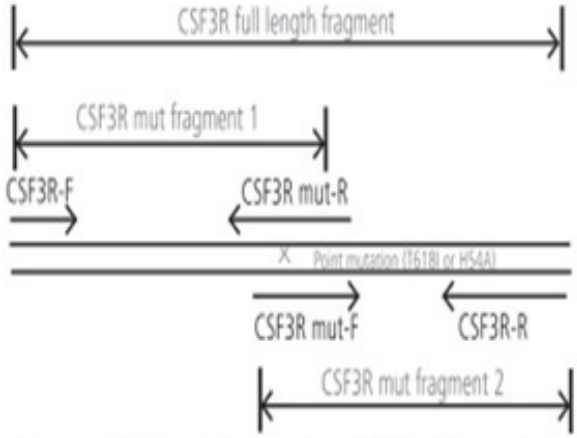


Figure 1. PCR Schematic presentation of CSF3R and its mutations.

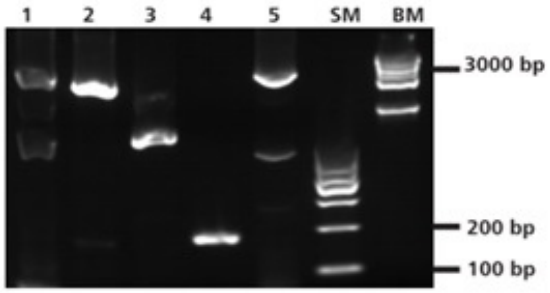


Figure 2. DNA fragment groups by PCR. Fragments of CSF3R were amplified by PCR and separated in 1.5% agarose gel for the following groups: (1) CSF3R-F/CSF3R-R (2500 bp), (2) CSF3R-F/T618I-R (1780 bp) (3) T618I-F/CSF3R-R (770 bp), (4) CSF3R-F/H54A-R (170 bp), (5) H54A-F/CSF3R-R (2330 bp). SM is the small-sized DNA marker (100-1000 bp) and BM is the big-sized DNA marker (1000-10000 bp).

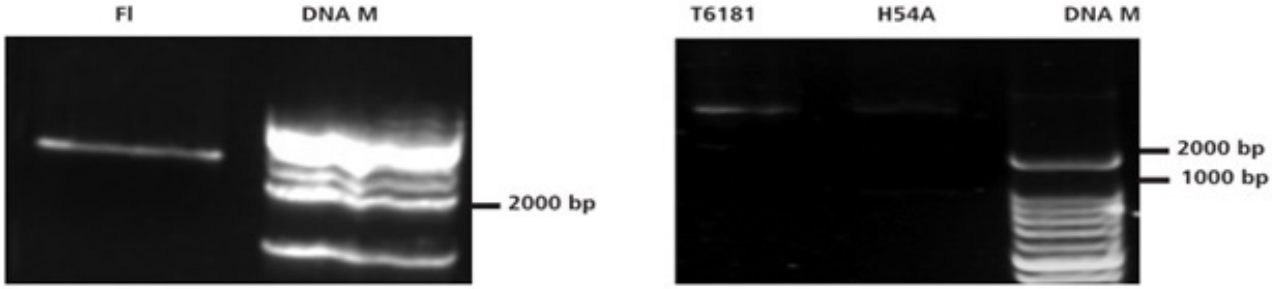


Figure 3. Agarose gel electrophoresis assessing the integrity of DNA fragments (CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A*) around 2500 bp. DNA M is the DNA marker.

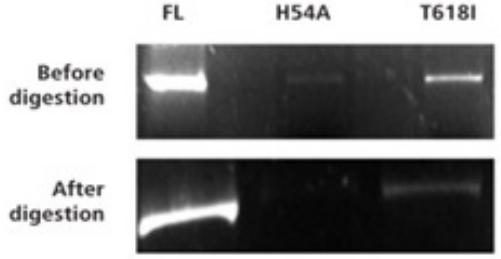


Figure 4. Restriction enzyme digestion test. Electrophoresis results with extracted CSF3R-*FL*, CSF3R-*T618I*, and CSF3R-*H54A* mutantafter restriction enzyme digestion with restriction enzymes EcoRI and NotI.

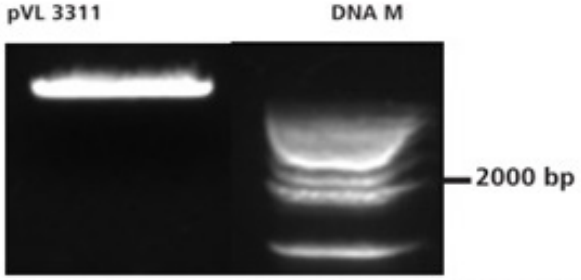


Figure 5. Electrophoresis results of extracted plasmid DNA after digestion with restriction enzymes EcoRI and NotI. DNA M is the DNA marker.

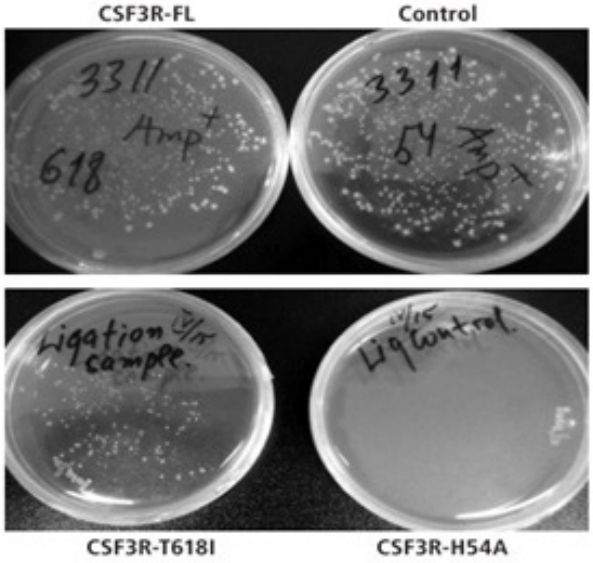


Figure 6. Plasmid transformation of CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A* compared to the control group.

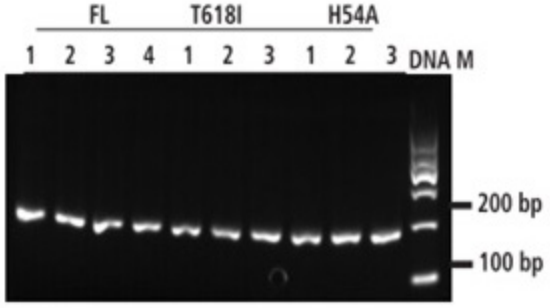


Figure 7. Ethidium bromide-stained agarose gel showing results of positive bands of the cloning CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A*. DNA M is the small-sized DNA marker.

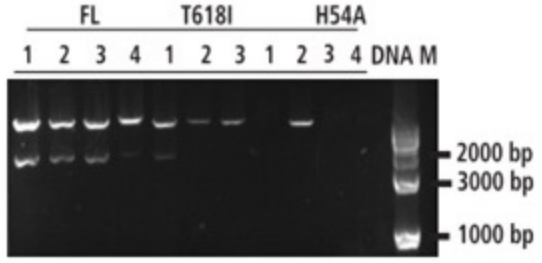


Figure 8. Plasmid molecules digested after cloning from the bacteria identified by restriction endonuclease analysis and visualized with agarose electrophoresis. DNA M is the big-sized DNA marker.

2.1 Culture of 293TN cell line

After seeding of cells, the cell density reached ~60-80% confluence at the time of transfection (Figure 9).

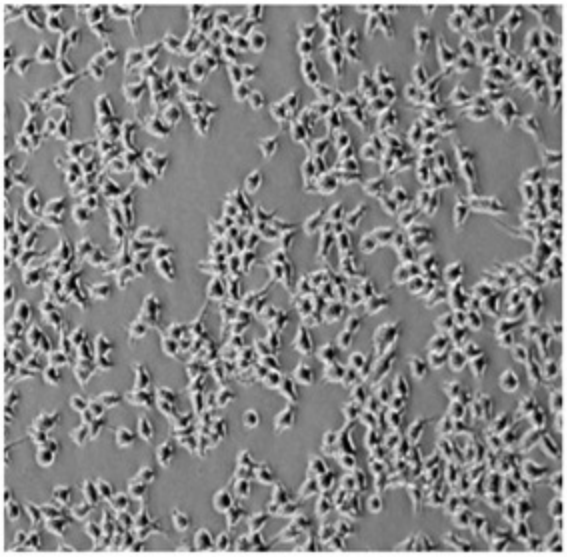


Figure 9. Phase contrast image of 293TN cells (×100). Cell density was between 60-80% confluence by the day of transfection.

2.2 Packaging HIV-based lentivector expression constructs

The GFP confirmed that the transfection experiment was successful (Figure 10).

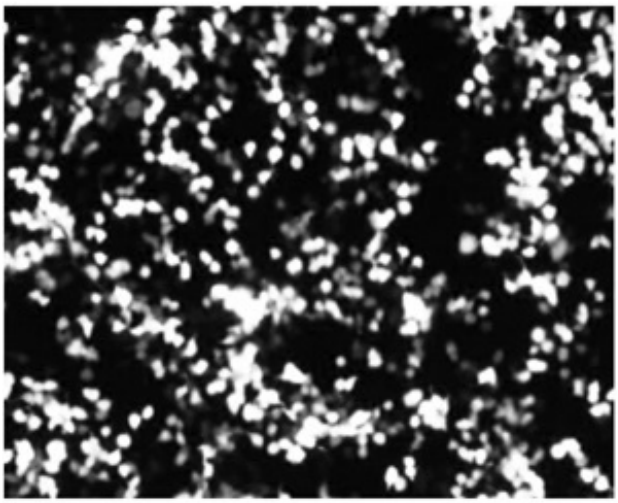


Figure 10. Fluorescent image of GFP-positive 293TN cells (×100) 24 hours after transfection with Lipofectamine, packaging plasmids and a lentivector construct encoding GFP.

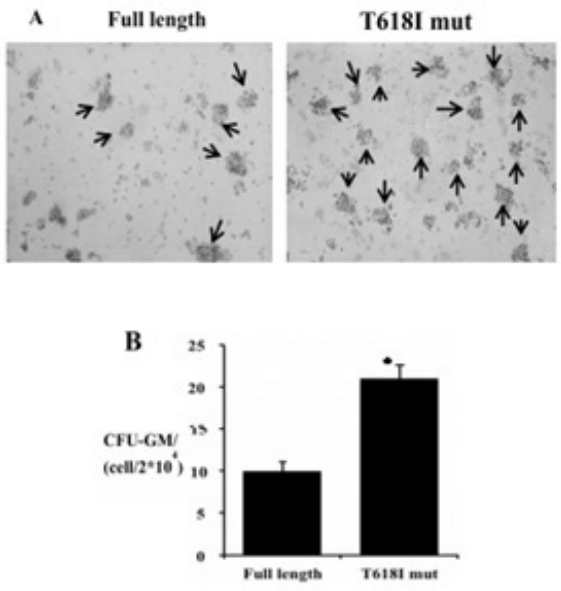


Figure 11. (A) Photograph of the CFU-GM colonies (×100) transformed by membrane proximal mutation (T618I mut) and full-length non-mutants (CSF3R-*FL*) detected using MethoCult™ 3534 media on the seventh day of culture. (B) Number of GM-CFU colonies after seven days of transforming. *p=0.0012.

Thus, we sought to determine whether CSF3R-H54A mutation has some correlation with molecular pathogenesis of CNL. Our experiment was based on that reported by Maxson et al. which showed results from interleukin-3–dependent Ba/F3 cells that were infected with murine retrovirus expressing wild-type CSF3R, membrane proximal mutations, or truncation mutations [5]. Uninfected parental Ba/ F3 cells and empty-vector infected Ba/ F3cells were used as controls [5]. Over a 10-day period, both classes of CSF3R mutations were capable of transforming Ba/F3 cells to interleukin-3–independent growth, and the membrane proximal mutations (T615A and T618I) transformed cells in this assay substantially faster than the truncation mutants (Q741X and S783fs). We successful-

ly obtained CSF3R-FL and CSF3R-T618I cloning, but we could not obtain cloning of CSF3R-H54A by whole methods of plasmid construction. Also, a lentiviral system was established and our transfection experiment of 293TN cells with packaging plasmids and the expression construct was confirmed under the positive control with GFP.

For the Mouse Colony Forming Unit Assay using MethoCultTM, our result showed that both of the of CSF3R mutations were capable of transforming murine colony forming cells, and the membrane proximal mutation (T618I) transformed CFU-GM colony number was more than the full length mutants (CSF3R-FL). This data was similar to previously published work and demonstrates that T618I mutation of CSF3R conferred the clonal advantage of CNL leukemia cells. Further studies are needed to prove the effects of the CSF3R mutations, including a novel mutation site CSF3R-H54A on the transduced murine bone marrow progenitor cells by using the colony forming cell assay.

Conclusion:

1. The establishment of the plasmid reconstruction, lentiviral packaging system and Mouse Colony Forming Unit Assay were done successfully.
2. We confirmed the transformation capacity of the CSF3R mutations, especially CSF3R-T618I was higher than CSF3R-FL. This result demonstrates that T618 mutation of CSF3R conferred the clonal advantage of CNL leukemia cells.
3. Further studies will be continued and are needed to prove the effects of the novel mutation site CSF3R-H54A on the transduced murine bone marrow progenitor cells by using CFC assay.

This thesis containing original research materials that have been published next peer reviewed journals with impact factor:

Oncology letter	1.9
Chinese Medical Journal	1.5

Original papers:

1. Otgonbat A, Zhao MF. Current strategies in the diagnosis and management of chronic neutrophilic leukemia. Chinese medical journal. 2014; 127(24):4258-62:10.3760/cma.j.issn. 0366-6999.20140305. Epub 2014/12/24. IF-1.077
2. Otgonbat Altangerel, Shannan Cao, Juanxia Meng, Peng Liu, Gong Haiyan, Yuanfu Xu, Ming Feng Zhao “Chronic neutrophilic leukemia with overexpression EVI-1 gene and concurrent CSF3R and SETBP1 mutation”. MS No: OL-5933-E134278. Received September 17, 2014; Accepted June 3, 2015 DOI: 10.3892/ol.2015.3485
3. Otgonbat A, Zhao MF, Wu Ri Mao “Study on the Mutation Sites of CSF3R Responsible for the Clonal Advantage of Chronic Neutrophilic Leukemia” “Central Asian Journal of Medical Sciences” Vol.2 №1, 2016 p37-42