Central Asian Journal of Medical Sciences
CAJMS

Original Article

Cent Asian J Med Sci. 2016 May;2(1):37-43

Study on the Mutation Sites of CSF3R Responsible for the Clonal Advantage of Chronic Neutrophilic Leukemia

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Submitted: January 10, 2016
Revised: March 29, 2016
Accepted: April 7, 2016

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Objectives: Chronic neutrophilic leukemia (CNL) is a rare, but distinct, entity within myeloproliferative neoplasms, characterized by splenomegaly, sustained mature neutrophilia, and absence of the Philadelphia chromosome. In 2013, oncogenic mutations in colony stimulation factor 3 receptor (CSF3R) were identified in a majority of patients with CNL and it was demonstrated that their downstream signaling was sensitive to known kinase inhibitors. This discovery was then validated with the demonstration of 100% CSF3R mutational frequency (predominately CSF3R-*T618I*) in strictly CNL as defined by the World Health Organization (WHO). In September 2012, a 67-year-old Chinese man was admitted to the First Central Clinical College of Tianjin Medical University (Tianjin, China) and presented as a case who had fulfilled the WHO diagnostic criteria for CNL with a oncogenic mutation in CSF3R-*T618I* and also an unreported novel mutation site of CSF3R-*H54A*. Thus, we sought to determine if CSF3R-*FL*, CSF3R-*T618I*, CSF3R-*H54A* mutations have some significance on the molecular pathogenesis of CNL. **Methods:** In this study, three main experiments were used including plasmid construction, lentiviral packaging system, and Mouse Colony-Forming Unit Assays Using MethoCult™. **Results:** The establishment of these three experiments was completed successfully. We confirmed the transformation capacity of the CSF3R mutations, especially that CSF3R-*T618I* was higher than CSF3R-*FL*. **Conclusion:** This result demonstrates that *T618I* mutation of CSF3R conferred the clonal advantage of CNL leukemia cells.

Keywords: Leukemia, Neutrophilic, Chronic; Transfection; Colony Stimulating Factor 3 Receptor (Granulocyte) Protein, Human; Genetic Transduction; Lentivirus

Introduction

Chronic neutrophilic leukemia (CNL) is a rare myeloproliferative neoplasm (MPN). Since the first description of CNL in 1920 [1], more than 150 cases were reported in the literature. The World Health Organization (WHO) recognizes CNL, as a MPN and, for the first time, provides recognized criteria to permit the operational classification of this poorly defined disease [2, 3]. Until recently, the molecular pathogenesis of CNL was unknown and the diagnosis was based on morphological aspects, clinical criteria and exclusion of known genetic entities like the Philadelphia translocation indicative of CML, or JAK2

mutations indicative of MPNs [4]. Recent discovery of high-frequency granulocyte-colony stimulating factor receptor (CSF3R) mutations in CNL identifies a new major diagnostic criterion, and lends more specificity to the WHO diagnostic criteria for CNL, which are variably applied in routine clinical practice. In 2013 Maxson et al. and Pardanani et al. identified granulocyte CSF3R mutations in 8 of 9 (89%) and in 13 of 13 (100%) patients with CNL, respectively [5, 6].

CSF3R mutations fall can be classified as nonsense or frameshift mutations that lead to premature truncation of the cytoplasmic tail of the receptor (truncation mutations) and point mutations in the extracellular domain of CSF3R (membrane proximal mutations). These nonsense or frameshift mutations truncate the cytoplasmic tail of CSF3R, impair its internalization, and alter its interactions with proteins such as SHP-1/2 and SOCS family members. These structural and functional alterations are thought to perturb the capacity of CSF3R to regulate granulocyte differentiation and to increase granulocytic proliferative capacity. The two types of CSF3R mutations may have differential susceptibility to classes of tyrosine kinase inhibitors, with CSF3R truncation mutations showing activation of SRC family–TNK2 kinase signaling and sensitivity to dasatinib and CSF3R membrane proximal mutations strongly activating the JAK/signal transducer and activator of transcription pathway and are sensitive to JAK kinase inhibitors such as ruxolitinib [7].

In follow-up, Pardanani et al. determined the frequency and specificity of CSF3R mutations in CNL and atypical chronic myeloid leukemia (aCML). This important study identified 14 CSF3R mutations in 13 patients, all of whom belonged to the group with either WHO-defined CNL (n = 12) or unconfirmed CNL (i.e., not meeting WHO criteria, n = 1), but none in the remaining categories. The majority were membrane proximal CSF3R mutations, most frequently CSF3R-*T618I* occurring in 10 (all with WHO-defined CNL), 2 had CSF3R-*M696T* mutations and 1 had a CSF3R-*I598I* mutation. There was a 100% CSF3R membrane proximal mutational frequency in WHO-defined CNL. One case co-expressed a truncating CSF3R mutation [6, 8]. The authors concluded that CSF3R-*T618I* is a highly sensitive and specific molecular marker for CNL and should be incorporated into current diagnostic criteria [6].

In September 2012, a 67-year-old Chinese man was admitted to the First Central Clinical College of Tianjin Medical University (Tianjin, China) and presented as a case who fulfilled the WHO diagnostic criteria for CNL with a novel mutation site of CSF3R [9]. In this case a membrane proximal mutation CSF3R-*T618I* was identified and also an unreported novel mutation site of CSF3R-*H54A* in the CD34+ and CD15+ cell fractions by sorting bone marrow samples (BD FACSAria™ III; BD Biosciences) using a PCR based DNA pyrosequencing method [9]. Therefore, the purpose of this study was to determine if CSF3R-*FL* (full length), CSF3R-*T618I*, and CSF3R- *H54A* mutations have some significance on the molecular pathogenesis of CNL.

Materials and 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, and pLP-1 gag pol were purified with a Plasmid Maxi Kit (Omega Bio-tek, USA) and digested by restriction enzymes (EcoRI and NotI, Thermo Scientific Co., Ltd, USA). The linearized vectors were purified from agarose gel using AxyPrepDNA Gel Extraction Kit (Axygen, Biosciences, USA) 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 also purified from agarose gels using AxyPrep DNA Gel Extraction Kits and digested by EcoRI. NotI was used to linearize the acceptor vector. Enzymatic reactions differed depending on the case. For the insert, 100–3000 ng of purified PCR product was digested by an appropriate amount of enzyme and 4 μL of 10x reaction buffer in 40 μl of final volume overnight at the appropriate temperature. For the vector, 2000–4000 ng of plasmid DNA was digested by an appropriate amount of enzyme and 2 μL of 10x reaction buffer in 20 μL of final volume for four hours at the appropriate temperature. When necessary the digested fragments were purified again, 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 [10, 11]. Chemically competent DH5α *Escherichia coli* bacteria were transformed with the products of the ligation reactions and were grown on Luria Bertrani agar plates containing the required antibiotic, such as ampicillin (Sigma-Aldrich, USA). One

day later, single colonies were picked from the plate, inoculated into and grown overnight in Luria Bertrani medium containing ampicillin. Transformed *E. coli* were selected from Luria Bertrani plates that contained amphicillin at 50 ng/mL (Amp) and the EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic, Canada) was used to isolate the plasmid from cultures of transformed cells. Plasmids were purified from the overnight cultures as above and tested by restriction mapping for the presence of the insert [12]. Selected clones were sequenced by Sanger sequencing.

2. Lentiviral packaging system

Three main components were used including 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), and 293TN producer cells. A total of $1x10^5$ 293TN cells were seeded per 10-cm^2 culture plate in 2-3 mL of culture medium containing Dulbecco's Modified Eagle's Medium supplemented with 4 mM L-glutamine, 4.5 g/L glucose, and fetal bovine serum (10%) without antibiotics. Cells were grown for 18-24 hours at 37°C with 5% CO_2 so that the cell density reached ~60-80% confluency at the time of transfection. We used a green fluorescent protein (GFP) as a positive control to confirm that the transfection experiment was successful. Then the cell culture supernatant was collected, which contained infectious pseudoviral particles [13, 14].

3. Transduction of pseudotyped viral particles into the primary murine cells

In the final step, the Mouse Colony Forming Unit Assay Using MethoCult™ (STEM CELL Technologies, USA) was used to assess the effects of CNL-associated oncogenes on the morphology and number of primary murine cells derived from bone marrow. For this purpose cells were transduced with either a control, which is without viral construct, or a construct expressing the oncogene of interest (CSF3R-*FL*, CSF3R-*T618I*).

Results

1. Plasmid construction of CSF3R-*FL*, CSF3R-*H54A* and CSF3R-*T618I* mutations

1.1 Obtained fragments of CSF3R-*FL*, CSF3R-*H54A* and CSF3R-*T618I* mutants

PCR diagrams of CSF3R and CSF3R mutations are shown in Figure 1. CSF3R-F/R primer was used to get the full length fragment (Figure 2, Lane 1), CSF3R-F/CSF3Rmut-R primers to get fragment 1 (Figure 2, Lane 2 for CSF3R-*T618I*, Lane 4 for CSF3R-*H54A*), and also CSF3Rmut-F/CSF3R-R primer to get fragment 2 (Figure 2, Lane 3 for CSF3R-*T618I*, Lane 5 for CSF3R-*H54A*). Then, fragment 1 and 2 were mixed as a template by using CSF3R-*F/R* as the primer to get the CSF3R mutant fragments (Figure 2).

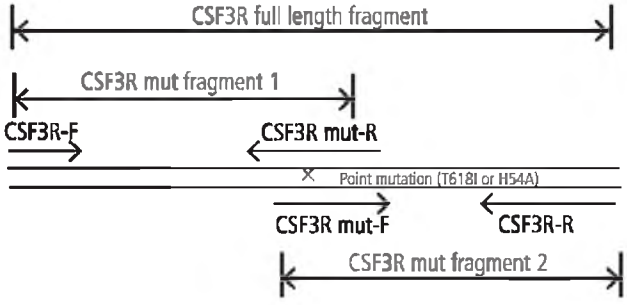

Figure 1. PCR Schematic presentation of CSF3R and its mutations.

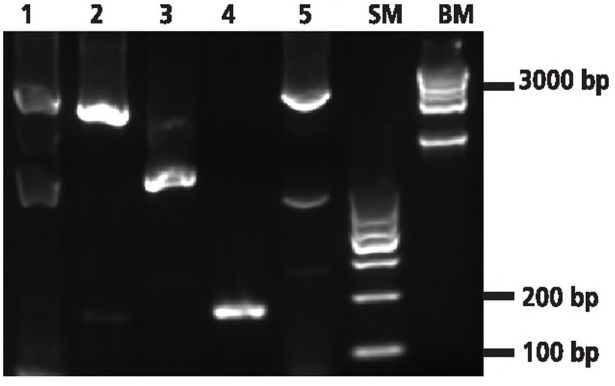

Figure 2. DNA fragment groups by PCR. Fragments of CSF3R were amplified by PCR and separated in 1.5% agarose gel for the following groups: (1) CSF3R-F/CSF3R-R (2500 bp), (2) CSF3R-F/T618I-R (1780 bp) (3) T618I-F/CSF3R-R (770 bp), (4) CSF3R-F/H54A-R (170 bp), (5) H54A-F/CSF3R-R (2330 bp). SM is the small-sized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

1.2 CSF3R full length purification

CSF3R fragments (2511bp) are not specific, so purification was performed by using a gel extraction kit and specific bands resulted (Figure 3).

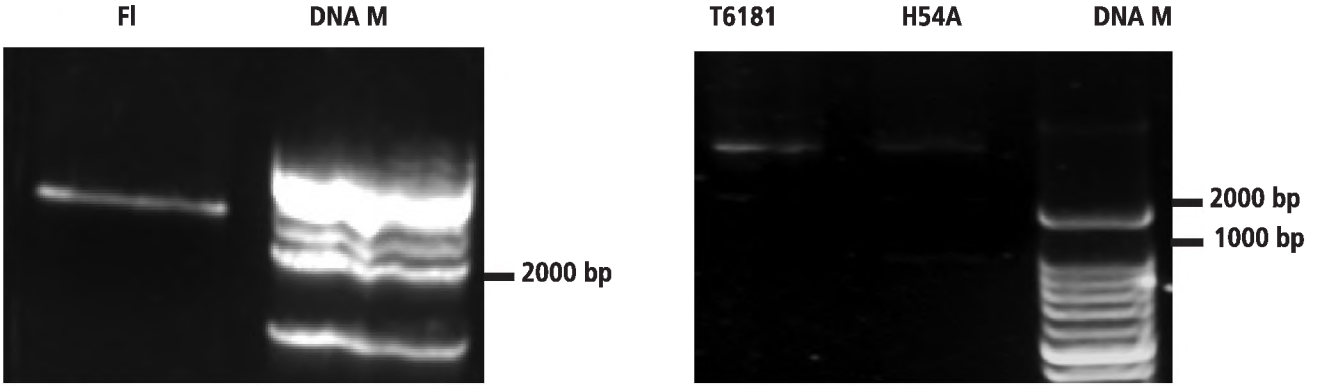

Figure 3. Agarose gel electrophoresis assessing the integrity of DNA fragments (CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A*) around 2500 bp. DNA M is the DNA marker.

1.3 Digestion for CSF3R, CSF3R-*T618I*, CSF3R-*H54A* mutant and plasmid

EcoRI and NotI restriction enzymes were used for double digestion. Before digestion, the quantity and quality of these three fragments was checked. After digestion, it was confirmed by using agarose gel electrophoresis that all fragments and plasmid were completely digested (Figures 4, 5).

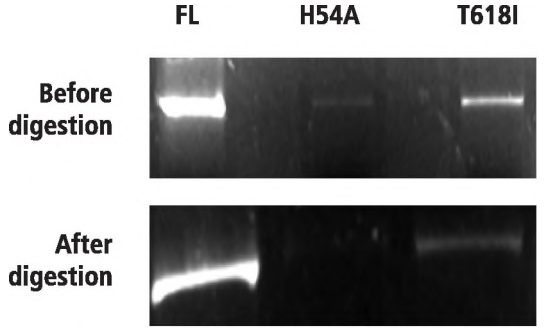

Figure 4. Restriction enzyme digestion test. Electrophoresis results with extracted CSF3R-*FL*, CSF3R-*T618I*, and CSF3R-*H54A* mutantafter restriction enzyme digestion with restriction enzymes EcoRI and NotI.

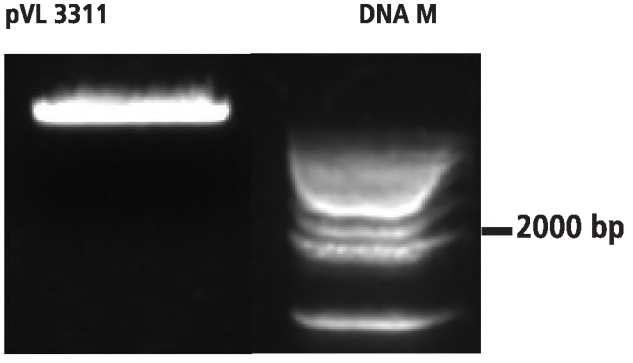

Figure 5. Electrophoresis results of extracted plasmid DNA after digestion with restriction enzymes EcoRI and NotI. DNA M is the DNA marker.

1.4 Ligation and transformation

After digestion, ligation and then transformation were performed. On the control group T4 ligase was not added resulting in no colony formation, but the experiment group formed several colonies (Figure 6). Our transformed colonies should have been of single colony growth. No bacterial contamination was expected.

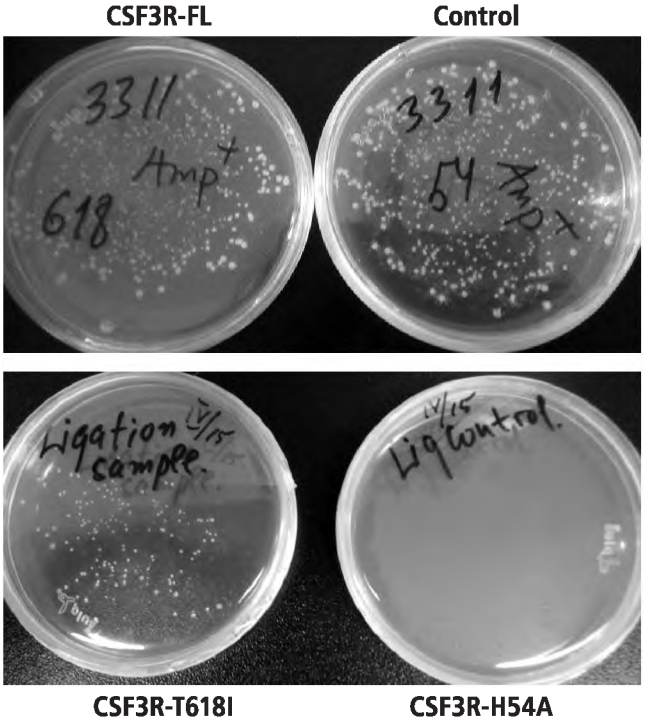

Figure 6. Plasmid transformation of CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A* compared to the control group.

1.5 Positive plasmid cloning

A single colony was selected and bacteria colony PCR was used to detect positive plasmids. All three of the cloning experiments demonstrated positive bands (Figure 7). For further confirmation, a double digestion by using restriction enzymes (EcoRI and NotI) was performed, meanwhile positive bands appeared on the CSF3R-*FL* and CSF3R-*T618I* cloning (Figure 8). The next step was completed at the same time, but an experiment was still done to obtain the CSF3R-*H54A* cloning.

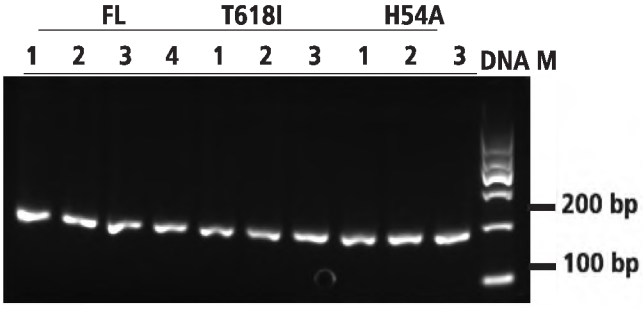

Figure 7. Ethidium bromide-stained agarose gel showing results of positive bands of the cloning CSF3R-*FL*, CSF3R-*T618I* and CSF3R-*H54A*. DNA M is the small-sized DNA marker.

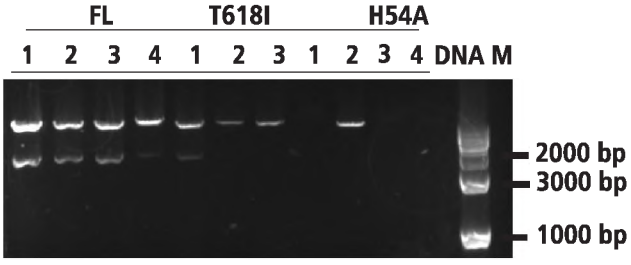

Figure 8. Plasmid molecules digested after cloning from the bacteria identified by restriction endonuclease analysis and visualized with agarose electrophoresis. DNA M is the big-sized DNA marker.

1.6 Identification of plasmid DNA sequencing

Further identification and sequence analysis were done with the help of Beijing Sheng Gong Company (Beijing, China) for two plasmids (CSF3R-*FL*, CSF3R-*T618I*). Their results agreed with our experiment.

2. Establishment of lentiviral system

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).

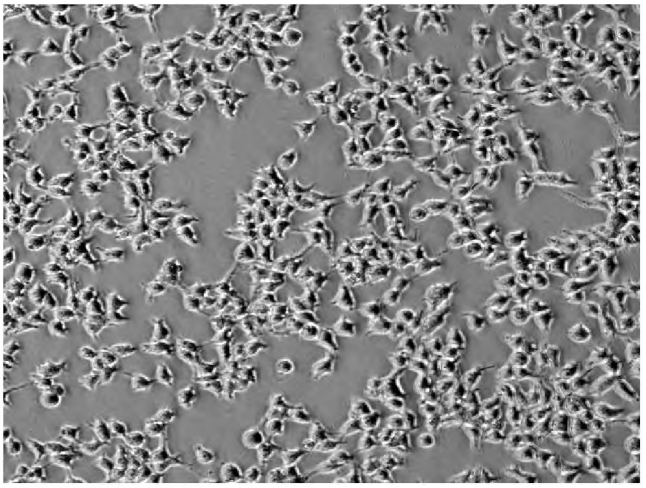

Figure 9. Phase contrast image of 293TN cells (×100). Cell density was between 60-80% confluence by the day of transfection.

2.2 Packaging HIV-based lentivector expression constructs

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

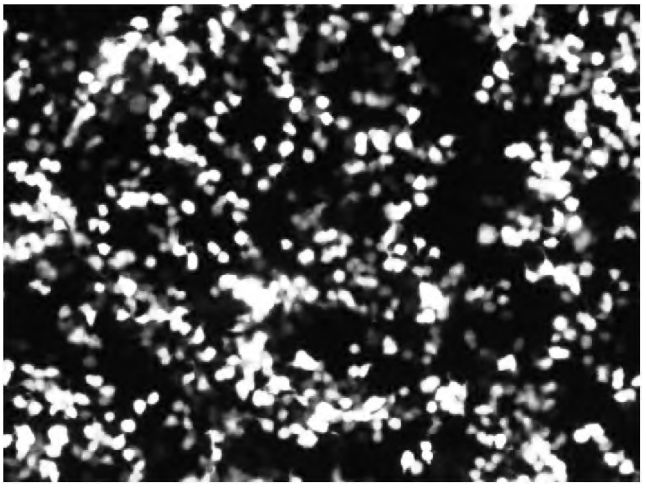

Figure 10. Fluorescent image of GFP-positive 293TN cells (×100) 24 hours after transfection with Lipofectamine, packaging plasmids and a lentivector construct encoding GFP.

3. Transduction of pseudotyped viral particles into the bone marrow murine progenitor cells

Both methods of CSF3R cloning were capable of transforming murine colony forming cells. After transforming, colony forming unit – granulocyte, monocyte (CFU-GM) colonies were counted manually by light microscopy seven days after plating (Figure 11). It was found that the membrane proximal mutation (*T618I*) transformed CFU-GM colony number was more than the full-length non-mutants (CSF3R-*FL*), which indicates that T618I mutation of CSF3R confers the clonal advantage of CNL leukemia cells (Figure 11).

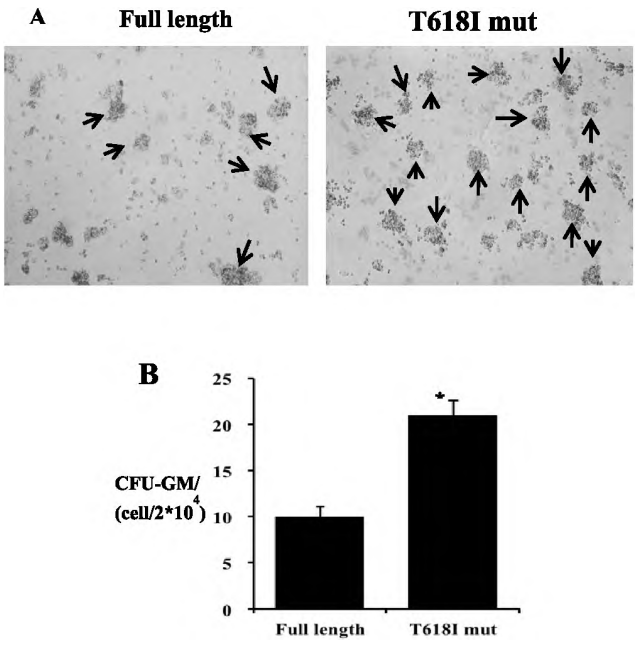

Figure 11. (A) Photograph of the CFU-GM colonies (×100) transformed by membrane proximal mutation (T618I mut) and full-length non-mutants (CSF3R-*FL*) detected using MethoCult™ 3534 media on the seventh day of culture. (B) Number of GM-CFU colonies after seven days of transforming. *p=0.0012.

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" [6]. 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 [6].

In our case, the membrane proximal mutation CSF3R-*T618I* was confirmed and also a previously unreported, novel mutation site *H54A* of CSF3R was identified. 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) [5]. We successfully 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 MethoCult™, 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 [5] 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.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

This study was supported by the National Natural Sciences Foundation of China (grant no. 81400092), the National Natural Sciences Foundation of Tianjin (grant no. 13JCYBJC23400) and the Science and Technique Foundation of Tianjin (grant no. 13KG106).

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