Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

Lack of FLT3-TKD835 gene mutation in toxicity of sulfur mustard in Iranian veterans

Hossein Ayatollahi [1], Mohammad Rafiee [2], Mohammad-Reza Keramati [1]*, Mahdi Balali-Mood [3], Ali Asgharzadeh [2], Mohammad Hadi Sadeghian [1], Maryam Sheikhi [1], Nafiseh Amini [1], Azam Moradi Zarmehri [2]

[1] Cancer Molecular Pathology Research Center, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[3] Medical Toxicology Research Center, Imam Reza Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Article type:
Original article

Article history:
Received: Jun 8, 2014
Accepted: Feb 1, 2015

Keywords:
FLT3-TKD835 mutation
Iran
Leukemia
Sulfur mustard

ABSTRACT

Objective(s): Sulfur mustard (SM) was used by the Iraqi army against the Iranian troops in the Iran-Iraq war from 1983–1988. This chemical gas affects different organs including the skin, lungs and the hematopoietic system. Any exposure to SM increases the risk of chromosomal breaking, hyperdiploidy and hypodiploidy. Studies have shown that the risk for acute myeloblastic and lymphoblastic leukemia increases in veterans exposed to SM. FLT3 mutations including ITD and TKD mutations had been observed in some cases of leukemia. Therefore, we aimed to investigate the frequency of FLT3-TKD835 mutations in the veterans exposed to SM agent.

Materials and Methods: We studied 42 patients who were exposed to SM during the war in Khorasan Razavi province, Mashhad, Iran in 2012. As control group, 30 healthy males were selected from first-degree relatives of the patients. For assessment of TKD835 mutation, DNA was extracted and RFLP-PCR was performed.

Results: Analysis of RFLP-PCR data showed no FLT-3 TKD mutation in any of the patients.

Conclusion: Although contact with SM can increase the risk of malignancy especially hematologic neoplasms, results of the study show that another mechanism of leukemogenesis, other than FLT3-TKD mutation, may be the reason for increased risk of leukemia in SM toxicity.

► *Please cite this article as:*
Ayatollahi H, Rafiee M, Keramati MR, Balali-Mood M, Asgharzadeh A, Sadeghian MH, Sheikhi M, Amini N, Moradi Zarmehri A. Lack of FLT3-TKD835 gene mutation in toxicity of sulfur mustard in Iranian veterans. Iran J Basic Med Sci 2015; 18:862-866.

Introduction

Sulfur mustard (SM), is a colorless or slightly yellow gas that was used by The Iraqi army against Iranian troops during the Iran-Iraq war between 1983 and 1988 (1-3). SM is one of blistering chemical warfare agents which was also used in the First World War (4, 5). This gas is responsible for over 100000 chemical casualties (4, 6). The target organs are the respiratory system, the skin, the eyes and the hematological system. The toxic effects on the respiratory system are very wide both in the acute phase and long term, inducing high morbidity and mortality. Its toxic effects on the skin include erythematic lesions, blisters, epidermolysis, necrosis of skin, respiratory dysfunctions and neuro-psychiatric complications (4). The eyes are more sensitive to SM, and the initial direct toxic effects are mostly irritation, leading to conjunctivitis, blepharospasm and keratitis (4, 7). SM toxic effects on the hematological system are very important, as the basis for the acute and long term complications including malignancies (2, 6, 8). Therefore, a wide range of SM toxicities occur following respiratory and dermal absorptions (7, 9-12).

SM is an alkylating agent which can alter the function of protein molecules. Its harmful effects take place by direct reaction with the nucleus of cells, in combination with various amino acids and production of toxic compounds from different tissue metabolites (4). Cytotoxic and mutagenic effects of SM can be explained through different biochemical reactions and structural changes in DNA (8). Short-term results of contact with the gas (first seven-weeks after exposure) are lymphopenia, neutropenia and thrombocytopenia (2, 4). IgM level increases at first but may decrease to normal level within six months. IgA levels also increase in some patients in early stages. The level of some complement proteins such as C3, C4 and CH50, was reported as normal during the first week to 6 months after exposure.

Studies on the short and long-term effects of SM

*Corresponding author: Mohammad Reza Keramati. Cancer Molecular Pathology Research Center, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel: +98-51-38521400; Fax: +98- 51-38591057; email: Keramatimr@mums.ac.ir

in Iranian patients with mild to moderate exposure revealed normal leukocyte count. However, in the severe cases there was a reduction in leukocyte count at the acute phase. There were also immature granulocytes and atypical lymphocytes in their blood (13). Flowcytometric studies in this group expressed some dysfunction of natural killer cells and decrease in absolute count of $CD56^+$ or $CD25^+/CD56^+$ cells in the long term (2).

According to genetic studies, contact with SM increases chromosomal breaking and hypodiploidy or hyperdiploidy (2). FLT-3 (FMS-Like Tyrosine kinase), is a proto-oncogene that belongs to type III of membrane tyrosine kinase receptors family. Mutation of FLT-3 is a type of gain of functional mutations (14). This receptor existed in immature hematopoietic cells such as blasts (15-17). As soon as the ligand of FLT-3 binds to this receptor, dimerization and autophosphorylation occur that are followed by creation of intracellular massages which cause activation and proliferation of target cells (18, 19). Various mutations occur in FLT-3 gene however, the most prevalent are internal tandem duplication (ITD) and tyrosine kinase domain 835 (TKD835) mutations. In ITD mutation, duplication occurs in the juxtamembrane domain. ITD has been reported in 16.5% of children with acute myeloblastic leukemia (AML), 20% of adults with AML, 34% of the elderly with AML, and also in 3% of myelodysplastic syndromes transformed to leukemia (20).

Uni-aminoacid mutations are commonly missense type change in FLT-3 structure (16, 17). The most prevalent substituent in these mutations is substitution of aspartic acid with tyrosine in D835 location at active loop (A-loop) of kinase domain. However, other substitutes such as Asp835Glu, Asp835Asn, Asp835Val and Asp835His have also been reported (21). FLT3 mutations are more frequent mutations in M3 and M4 subgroups of AML (22). D835 mutation causes persistent phosphorylation even when there are not any ligands (23). According to several studies, TKD mutation is reported in 5.8-7.7% of adults with AML (17, 23, 24). On the other hand, risk of acute myelocytic leukemia increases in persons exposed to SM (8). Considering the role of SM toxicity in pathogenesis of neoplasms especially hematopoietic neoplasms and also the role of FLT-3 TKD mutation in these neoplasms, we evaluated this mutation in the patients with previous SM exposure during the Iran-Iraq war.

Materials and Methods

This case control study was implemented in Medical Toxicology Research Center of Imam Reza Hospital and Molecular Pathology and Cytogenetic Research Center of Ghaem Hospital (two major teaching hospitals), Mashhad University of Medical Sciences, in collaboration with the veterans (Janbazan) foundation of Khorasan Razavi province, Mashhad, Iran in 2012. The ethical committee of Mashhad University of Medical Sciences approved the research protocol and a written informed consent was taken from each participant. Janbazan foundation has all the documents and evidences about SM intoxication in these patients including medical history and their treatment process. The patients entered in this study have been suffering from different SM toxicity complications especially in the lungs, the skin and eyes since exposure (1983-1988). The severity grading of SM toxicity and long term complications of the veterans were assessed regularly every few years by a medical committee of the Janbazan foundation. The patients with <25% disabilities were considered as mild, 25–50% as moderate and >50% as severe cases. Veterans with more than 25% percent disability were considered as having moderate to severe SM complications and were included in this study. Patients with suspected history of exposure to any other chemical agents were excluded from the study. Control group consisted of 30 adult healthy males from the first-degree relatives of the patients living in similar conditions with that of the case group regarding place of residence and rate of exposure to possible chemical agents in the environment.

Ten milliliters of peripheral blood with K2-EDTA anticoagulant was taken from each participant. Initially, complete blood count (CBC) was performed on all samples (by Sysmex KX-21N, Japan) and then DNA extraction was performed (by Qiagen kit, Iran) and purity of extracted DNA was measured by Nanodrop set which was satisfactory. To detect D835uni-amino acid mutation, PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) was performed by ABI thermo cycler (USA). Primers for PCR were forward primer: 5′-CCGCCAGGAACGTGCTTG-3′ and reverse primer: 5′-GCAGCCTCACATTGCCCC-3′. Primers were designed by Blast software at ncbi.nlm.nih.gov, and were synthesized by Metabion Co. (Germany). After PCR and obtaining 114 bp sequences, for detecting D835 mutation based on RFLP procedure, products were incubated overnight at 37 ˚C with the EcoRV restriction enzyme (Fermentas). A patient with FLT3-TKD835 mutation as a positive control was used. Then PCR products were run in agarose gel 2.5% and stained by ethidium bromide. In FLT3-TKD mutation, restriction enzyme could not break the products but in wild type FLT3, products break down into two different sizes of sequences by EcoRV restriction enzyme.

Statistical analysis

Collected data were analyzed using SPSS software, version 11.5. Initially, descriptive and then comparative analysis of variables were performed. The means of continuous variables were compared by using independent sample t-test or its non-

Table 1. Comparison of hematologic values between the patients who were exposed to sulfur mustard during the Iran-Iraq conflict and a control group

Parameter	Patient group Mean±SD (Range)	Control group Mean±SD (Range)	*P-value*
WBC ($\times 10^9$/l)	6.61 ± 1.42 (4.2-10)	6.29 ± 1.30 (3.8-8.8)	0.338
RBC ($\times 10^{12}$/l)	5.62 ± 0.87 (4.61-9.96)	5.70 ± 0.50 (4.87-7.24)	0.650
Hemoglobin (gr/dl)	15.9 ± 1.58 (12.7-21)	16.2 ± 0.95 (14.6-18.2)	0.382
Hematocrit (%)	46.0 ± 4.10 (38.3-59.7)	46.5 ± 2.99 (41.3-53.5)	0.583
Platelet ($\times 10^9$/l)	237 ± 49.68 (166-388)	241 ± 50.54 (161-348)	0.737

parametric equivalent, Mann-Whitney test. A *P-value* ≤ 0.05 was considered significant.

Results

After the exclusion, 42 male patients and 30 healthy males were studied. The ages of patients ranged from 40 to 60 years and in the control group it was 20 to 41 years. Results of CBC analysis of patients and control groups are shown in Table 1. These values were not significantly different between the two groups.

Qualities of extracted DNA from all samples were good, as concentrations of them in Nanodrop were 400–500 ngr/µl. For all patients and the control subjects, RFLP was performed. However, as shown in Figure 1, FLT-3 TKD mutation was observed neither in the patient nor in the control groups.

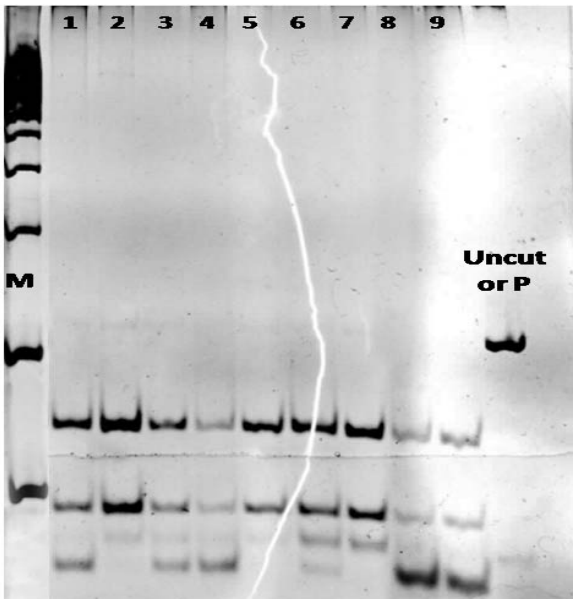

Figure 1. Electrophoresis of RFLP products in patients who were exposed to sulfur mustard during the Iran-Iraq conflict. None of our patients (1, 2… 9) revealed FLT3-TKD mutation, because the EcoRV enzyme could digest the FLT3 gene sequences to two 68 bp and 46 bp pieces. In the positive control sample (uncut or P) the enzyme could not digest this and the sequence remained 114 bp. M: marker

Discussion

A neoplasm may occur by the clonal proliferation of precursor cells with some genetic damages. Proto-oncogenes, tumor suppressor genes, genes involved in DNA repair, and apoptosis are four groups of normal regulatory genes that are the basic targets of these damages. Genetic alterations such as mutations in proto-oncogenes, can be formed in exposure to toxic chemical materials, and formed oncogenes that evolve can proliferate in cells in the absence of growth stimulation (25).

Hematopoiesis is controlled by several growth factors and many of them perform their functions through binding to tyrosine kinase receptors such as FLT3 receptor. FLT-3 mutation is one of the prevalent mutations in leukemia that results in uncontrollable proliferation of cells and decreases the prognosis. These mutations have an important role in hematopoietic progenitor cell proliferation and survival and in leukemia pathogenesis; it is mutated in about 1/3 of AML patients. The rate of TKD mutation in leukemia is much lower than ITD mutation (16, 17, 26, 27). According to several studies, TKD mutation is reported in 5.8–7.7% of adult AMLs (17, 23, 24). Besides, the involvement of different mutations in leukemia, some chromosomal abnormalities such as trisomy 8, monosomy 7 are also important (28). One of late toxic effect of SM is decreased numbers of natural killer cells in blood that can last for decades after the exposure. This cell-mediated immunological disorder can be associated with increased risk of malignancies in these patients decades after the exposure (29).

Although the studies showed that SM toxicity could have cause DNA damages and increase the potential risk of tumorigenesis, we did not detect any FLT-3 mutation in the patients after about 25–30 years of SM exposure. Tumorogenesis is a multistep pass way resulting from the gathering of multiple mutations over a period of time (30). Absence of observation of this mutation in this study may be due to occurrence of this mutation late in mutagenesis pass way or due to other genetic damage apart from FLT-3 mutation. In addition, due to lapse of a long time after SM exposure some of these patients have died of secondary neoplastic disorders probably in

background of some genetic mutations and this can be another reason why we could not detect the mutation in the remaining patients.

A study of ploidy on patients who suffered from SM toxicity, revealed aneuploidy in peripheral blood lymphocytes that can be a reason for increased risk of tumorigenesis in these patients (2). Serum cytokine profiles change in SM toxicity (31). After SM exposure, IL-6 and GM-CSF expression increase as a short term result of SM toxicity (32). IL-6 can inhibit programmed cell death in many tumor cell (33). Also, high levels of the GM-CSF receptors express in leukemic cells (34). These effects of SM, can lead to alterations in myeloid cells and promote their proliferation.

Previous study also showed that expression of 19 genes correlated with apoptosis, transcriptional factors, cell cycle, inflammation, oncogenes, and tumor suppressors, increased after dermal contact with SM in mice (35). Changes in genes that are related to proliferation, growth and differentiation have been observed in leukemia. Decrease in expression of anti-inflammatory cytokines can increase the number of myeloid cells as in AML. SM can reduce the levels of transforming growth factors β1 and β2 as anti-inflammatory cytokines (36). These facts show that SM can increase risk of leukemia by different mechanisms. Therefore, it is also necessary to survey other mutations in these patients.

CBC parameters in the patients and control groups were similar. Although cytopenia is one of the laboratory manifestations of chronic SM toxicity, this similarity may be due to milder exposure of these patients to SM, which has also caused greater survival of the patients.

Conclusion

Although SM exposure can increase the risk of malignancy especially hematologic neoplasms, we did not detect any FLT-3 TKD mutations in our patients. This means that other mechanisms of leukemogenesis may be the reason for increased risk of leukemia in SM toxicity. Considering the importance of health of the SM veterans and value of prophylactic care, we suggest the assessment of other genetic aspects of tumorigenesis in these patients.

Acknowledgment

The authors are grateful to the Vice-president for Research, Mashhad University of Medical Sciences, Mashhad,Iran for financial support of this study and the Janbazan foundation of Khorasan Razavi for great cooperation.

References

1. Ghanei M, Harandi AA. Long term consequences from exposure to sulfur mustard: a review. Inhal Toxicol 2007; 19:451-456.
2. Hassan ZM, Ebtekar M, Ghanei M, Taghikhani M, Noori Daloii M, Ghazanfari T. Immunobiological consequences of sulfur mustard contamination. Iran J Allergy Asthma Immunol 2006; 5:101-108.
3. Sanjarmoosavi N, Sanjarmoosavi N, Shahsavan M, Hassanzadeh-Nazarabadi M. Teratogenic effects of sulfur mustard on mice fetuses. Iran J Basic Med Sci 2012; 15:853-859.
4. Balali-Mood M, Hefazi M. The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. Fundam Clin Pharmacol 2005; 19:297-315.
5. Robinson JP. Public health response to biological and chemical weapons: WHO guidance: World Health Organization; 2004.
6. Mahmoudi M, Hefazi M, Rastin M, Balali-Mood M. Long-term hematological and immunological complications of sulfur mustard poisoning in Iranian veterans. Int Immunopharmacol 2005; 5:1479-1485.
7. Balali-Mood M. Clinical and paraclinical findings in 233 patients with sulfur mustard poisoning. 1986.
8. Balali-Mood M, Hefazi M. Comparison of early and late toxic effects of sulfur mustard in Iranian veterans. Basic Clin Pharmacol Toxicol 2006; 99:273-282.
9. Balali-Mood M, Balali-Mood B. Sulphur mustard poisoning and its complications in Iranian veterans. Iran J Med Sci 2009; 34:155-171.
10. Balali-Mood M, Mousavi S, Balali-Mood B. Chronic health effects of sulphur mustard exposure with special reference to Iranian veterans. Emerg Health Threats J 2008;1.
11. Hefazi M. The clinical toxicology of sulfur mustard. Arch Iran Med 2005; 8:162-179.
12. Sidell FR, Takafuji ET, Franz DR. Medical aspects of chemical and biological warfare. office of the surgeon general (army) falls church va; 1997.
13. Keramati MR, Balali-Mood M, Mousavi SR, Sadeghi M, Riahi-Zanjani B. Biochemical and hematological findings of Khorasan veterans 23 years after sulfur mustard exposure. J Res Med Sci 2013; 18:855-859.
14. Small D. FLT3 mutations: biology and treatment. ASH Educ Program Book 2006; 2006:178-184.
15. Liang D, Shih L, Hung I, Yang C, Chen S, Jaing T, *et al.* FLT3-TKD mutation in childhood acute myeloid leukemia. Leukemia 2003; 17:883-886.
16. Moreno I, Martín G, Bolufer P, Barragán E, Rueda E, Román J, *et al.* Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. Haematologica 2003; 88:19-24.
17. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, *et al.* Analysis of FLT3-activating mutations in 979 patients with *acute* myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis Presented in part at the 42nd Annual Meeting of the American Society of Hematology, December 1-5, 2000, San Francisco, CA (abstract 2334). Blood 2002; 99:4326-4335.
18. Heldin C-H. Dimerization of cell surface receptors in signal transduction. Cell 1995; 80:213-223.
19. Turner AM, Lin NL, Issarachai S, Lyman SD, Broudy V. FLT3 receptor expression on the surface of normal

and malignant human hematopoietic cells. Blood 1996; 88:3383-90.
20. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, *et al.* Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 1997; 11:1605-1609.
21. Shih L-Y, Huang C-F, Wu J-H, Wang P-N, Lin T-L, Dunn P, *et al.* Heterogeneous patterns of FLT3 Asp835 mutations in relapsed de Novo acute myeloid leukemia A comparative analysis of 120 paired diagnostic and relapse bone marrow samples. Clin Cancer Res 2004; 10:1326-1332.
22. Wang L, Lin D, Zhang X, Chen S, Wang M, Wang J. Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients. Leuk Res 2005; 29:1393-1398.
23. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, *et al*. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 2001; 97:2434-2439.
24. Abu-Duhier F, Goodeve A, Wilson G, Care R, Peake I, Reilly J. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. Br J Haematol 2001; 113:983-988.
25. Kooshyar MM, Ayatollahi H, Keramati MR, Sadeghian MH, Miri M, Sheikhi M. Lack of KRAS gene mutations in chronic myeloid leukemia in Iran. Asian Pac J Cancer prev 2013; 14:6653-6656.
26. Sheikhha MH, Awan A, Tobal K, Liu Yin JA. Prognostic significance of FLT3 ITD and D835 mutations in AML patients. Hematol J 2003; 4:41-46.
27. Sheikhi M, Zaker F, Javadi GR, Hashemi M, Razmkhah F. Assesment of FLT3-gene mutations among children with acute leukemia. Med Sci J Islam Azad Univ Tehran Med Branch 2009; 19:230-235.
28. Rees J. Aneuploidy in Acute Myeloid Leukemia. Tumor aneuploidy: Springer; 1985. p. 25-28.
29. Balali-Mood M, Hefazi M. Comparison of early and late toxic effects of sulfur mustard in Iranian veterans. Basic Clin Pharmacol Toxicol 2006; 99:273-282.
30. Stricker TP, Kumar V. Neoplasia. In: Kumar V, Abbas AK, Fausto N, Aster JC, editors. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Philadelphia: Saunders Elsevier; 2010. p. 259-320.
31. Riahi-Zanjani B, Balali-Mood M, Mousavi SR, Karimi G, Sadeghi M, Shirmast E, *et al.* Serum cytokine profiles of Khorasan veterans 23 years after sulfur mustard exposure. Cytokine 2014; 70:161-164.
32. Sabourin CL, Petrali JP, Casillas RP. Alterations in inflammatory cytokine gene expression in sulfur mustard–exposed mouse skin. J Bochem Mol Toxicol 2000; 14:291-302.
33. Isomoto H, Kobayashi S, Werneburg NW, Bronk SF, Guicciardi ME, Frank DA, *et al.* Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. Hepatology 2005; 42:1329-1338.
34. Budel LM, Touw IP, Delwel R, Clark SC, Lowenberg B. Interleukin-3 and granulocyte-monocyte colony-stimulating factor receptors on human acute myelocytic leukemia cells and relationship to the proliferative response. Blood 1989; 74:565-571.
35. Rogers JV, Choi YW, Kiser RC, Babin MC, Casillas RP, Schlager JJ, *et al.* Microarray analysis of gene expression in murine skin exposed to sulfur mustard. J Biochem Mol Toxicol 2005; 18:289-299.
36. Wu H, Li P, Shao N, Ma J, Ji M, Sun X, *et al.* Aberrant expression of Treg-associated cytokine IL-35 along with IL-10 and TGF-β in acute myeloid leukemia. Oncol Lett. 2012; 3:1119-1123.