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RESEARCH ARTICLE

MULTIPLE MYELOMA ONCOGENE-1 (MUM-1) DETECTION BY FLOW CYTOMETRY TECHNIQUE IN SOME B-CELL MALIGNANCIES.

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Abstract

Background: Multiple Myeloma Oncogene 1 (MUM1)/ interferon regulatory factor 4 (IRF4) is a member of the interferon regulatory factor family of transcription factors. It was thought to play an important role in the pre-B cell development, receptor editing, germinal center reaction as well as plasma cell generation. This retrospective study aimed to assess MUM1/ IRF4 diagnostic value as a step for further studies to determine its significance in therapeutic strategies for B cell malignancies.

Methods: A total of 60 individuals were enrolled in this study. They were divided into two groups; the patient group, which included 40 newly diagnosed B cell malignancies patients (40 to 80 years old), 24 (60%) males and 16 (40%) females. They included 20 cases of Multiple Myeloma, 10 cases of Chronic lymphocytic Leukemia and 10 cases of Non-Hodgkin Lymphoma. The control group included 20 random subjects of cases other than B cell malignancies. Patients were diagnosed by flow cytometric immunophenotyping using routine panel of monoclonal antibodies for B cell malignancies followed by MUM1/IRF4 detection using anti MUM1/IRF4 monoclonal antibodies Kit (MACS, Milteny Biotec Inc., USA).

Results: MUM1/IRF4 showed highest statistical difference among multiple myeloma group than among Chronic Lymphocytic Leukemia group and least among Non-Hodgkin Lymphoma and control groups. Average expressions were 93.1%, 24.2%, 6.5% and 0.6% respectively.

Conclusion: MUM1/IRF4 had a significant role as a diagnostic marker in multiple myeloma.

Introduction:-

Multiple myeloma oncogene 1 (MUM1)/ interferon regulatory factor 4 (IRF4) gene is responsible for encoding the transcriptional factor IRF4 that plays an important role in the development of lymphoid cells. Transcription factors control DNA transcription into RNA sequences, by recognizing specific DNA sequences; they facilitate or inhibit transcription, which is the first step of gene expression. [1]

In B cells specifically, IRF4 was found to be expressed at different stages to control important steps affecting their differentiation, transformation and function. [2]

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Consistent with the crucial involvement of IRF4 in development of B cells, deregulated expression of IRF4 was suspected to be associated with pathogenesis of several B cell neoplasms and diseases. In B cell derived Chronic lymphocytic leukemia (CLL); IRF4 is thought to function as a tumor suppressor. However, in multiple myeloma (MM); it is thought to act as a survival factor. [3]

This retrospective study was conducted to evaluate the significance of MUM1/IRF4 as a phenotypic marker in some B cell malignancies, in a step for further studying its role as a therapeutic target.

Subjects and Methods:-

Study population

A total of 60 individuals were enrolled in this study. They were divided into two main groups; the patient group, which included 40 patients newly diagnosed with B cell malignancies. Their ages ranged from 40 to 80 years old, with mean of 59.9 years. They were 24 (60%) males and 16 (40%) females with a male to female ratio of 1.5:1. The patient group was further divided into three subgroups as follows; 20 cases of MM, 10 cases of CLL and 10 cases of NHL.

The control group, which included 20 random subjects of cases other than B cell malignancies. Their ages ranged from 42 to 80 years old with mean of 59.7 years. They were 12 (60%) males and 8 (40%) females, with male to female ratio 1.5:1.

Immunophenotyping

Venous blood samples and/or BM aspirates were collected, in Ethylene Diamine Tetra Acetic acid (EDTA) containing tubes. These samples were used for performing immunophenotyping (IPT) for diagnosis of cases followed by detection of MUM1/IRF4^{+} cells.

For proper enumeration and identification of IRF4+ cells; an anti-IRF-4 antibodies Kit (MACS, Milteny Biotec Inc., USA) was utilized, according to its manufacturer instructions. MACSR Cell Signaling Antibodies (Monoclonal Anti-IRF-4 antibodies) were included in the kit and the antibodies were PE conjugated.

Preparation of permeabilization-buffer was done using phosphate-buffered saline (PBS) solution with pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376, Milteny Biotec Inc., USA) 1:20 with auto MACS Rinsing Solution (# 130-091-222, Milteny Biotec Inc., USA). Then it was kept at cold temperature (2−8 °C) till usage.

For cell fixation and permeabilization; Cell Signaling Buffer Set A (# 130-100-827, Milteny Biotec Inc., USA) was utilized to detect the intracellular proteins by flow cytometry technique. Permeabilization Buffer A was then allowed to cool to –20 °C before usage because higher temperatures or longer incubation times may lead to non-specific cell labeling. [4,5]

The cells were fixed directly by adding 4 volumes of the cell suspension to 1 volume of Inside Fix. 250 µL Inside Fix was added to 1 mL cells suspension with 10^6 cells. The mixture was incubated at room temperature (18–25 °C) for 10 minutes. Then it was centrifuged at 500×g for 5 minutes at 4 °C, and the supernatant was aspirated. Vortex was done to loosen the cells.

The cells were permeabilized by adding 1 mL of Permeabilization Buffer A per 10^6 cells. The mixture was vortexed and kept on ice for 30 minutes then rinsed by 3 mL of buffer A.

Samples were centrifuged at 500×g for 5 minutes at 4°C, then the supernatant was aspirated. The cells were washed again by adding 4 mL of buffer A and centrifuged at 500×g for another 5 minutes at 4°C. The supernatant was aspirated.

The cells were resuspended up to 10^7 nucleated cells per 100 µL buffer A and 10 µL of the MACS Cell Signaling Antibody was added.

The suspension was incubated for 30 minutes in the dark at room temperature. After which, the cells were washed by adding 3 mL buffer A and centrifuged at 500×g for 5 minutes. Resuspension in 0.5 mL buffer A for analysis by flow cytometry was done and the mixture was stored at 2–8 °C in the dark until analysis.

For immunofluorescent staining, $CD19^+$ B cells were gated in cases of CLL and NHL, while, $CD138^+$ Plasma cells were gated in cases of MM. The cells were then fixed and permeabilized then stained with Anti-IRF-4 antibodies and analyzed by flow cytometry using EPICS XL Flow Cytometer, Coulter, USA.

Statistical methods:-

The collected data were statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp., Chicago, USA, 2009.

Descriptive statistics were done for quantitative data as minimum & maximum of the range as well as mean ± SD (standard deviation) for quantitative normally distributed data, while it was done for qualitative data as number and percentage.

Inferential analyses were done for quantitative variables using Shapiro-Wilk test for normality testing, independent t-test in cases of two independent groups with normally distributed data and ANOVA test with post hoc Tukey test for more than two independent groups with normally distributed data. In qualitative data, inferential analyses for independent variables were done using Chi square test for differences between proportions and Fisher's Exact test for variables with small expected numbers. While correlations were done using Pearson correlation for numerical normally distributed data and using partial to control age when required. ROC curve was used to evaluate the performance of different tests differentiate between certain groups. The level of significance was taken at P value < 0.050 is significant and < 0.001 is highly significant, otherwise is non-significant.

Results:-

Table 1 represents Bone marrow immunophenotypic markers among the multiple myeloma group. The highest expression was detected for the immunophenotypic markers CD 38, CD 138 and CD 11b which were expressed in 20 (100%) of patients, while the lowest was detected for **λ** and CD 19 which were expressed in 2 (10%) of patients.

Table 1:-Bone marrow immunophenotypic markers among multiple myeloma group.

BM findings		**Mean±SD**	**Range**
Immunophenotypic markers		**(N=20)**	**%**
CD11b		20	100.0
CD19		2	10.0
CD20		5	25.0
CD38		20	100.0
CD45		7	35.0
CD56		14	70.0
CD117		4	20.0
CD138		20	100.0
κ/λ	**K**	18	90.0
	λ	2	10.0

Table 2 represents the comparison between immunophenotypic markers regarding MUM1/IRF4 among multiple myeloma group. It showed no significant differences.

Table 2:-Comparison between immunophenotypic markers regarding MUM1/IRF4 among multiple myeloma group.

Marker	**Present**		**Absent**		**P**
	(N=20)	**Mean±SD**	**(N=20)**	**Mean±SD**	
CD19	2	72.0±3.9	18	13.3±6.3	0.740
CD20	5	92.2±4.7	15	13.4±6.4	0.698
CD45	7	35.0±6.5	13	15.2±4.5	0.276
CD56	14	82.4±6.2	6	14.7±5.5	0.446

CD117	4	64.8±6.4	16	14.3±4.0	0.673

^Independent t-test

Table 3 represents bone marrow/ peripheral blood immunophenotypic markers among the CLL group. It showed highest expression for CD5, CD19 and CD 23 in 10 (100%) of patients, while the lowest was for **λ** in 3 (30%) of patients.

Table 3:- Bone marrow/peripheral blood immunophenotypic markers among the CLL group.

Variables		**Mean±SD**	**Range**
Immunophenotypic markers		**(N=10)**	**%**
CD5		10	100.0
CD19		10	100.0
CD23		10	100.0
CD20 (dim)		9	90.0
CD38		4	40.0
CD79b (dim)		8	80.0
sIgM (dim)		9	90.0
κ/λ	**K**	7	70.0
	λ	3	30.0

Table 4 represents the comparison between immunophenotypic markers regarding MUM1/IRF4 among CLL group showed that there were no significant differences between the immunophenotypic markers expression regarding MUM1/IRF4 among CLL group.

Table 4:- Comparison between immunophenotypic markers regarding MUM1/IRF4 among CLL group.

Marker	**Present**		**Absent**		**P**
	(N=10)	**Mean±SD**	**(N=10)**	**Mean±SD**	
CD38	4	29.3±4.0	6	12.9±3.6	0.706
CD79b	8	34.2±31.5	2	15.8±2.1	0.642
K	7	30.1±3.9	3	10.4±1.0	0.389
λ	3	32.6±2.2	7	18.5±2.7	0.211

^Independent t-test

Table 5 represents bone marrow findings and immunophenotypic markers among NHL group. It showed highest expression for CD 19, CD 20 and CD 79b in 10 (100%) of patients, while the lowest was for CD 103 in 1 (10%) of patients.

Table 5:- Bone marrow immunophenotypic markers among NHL group.

Variables		**Mean±SD**	**Range**
Immunophenotypic markers		**(N=10)**	**%**
CD10		1	10.0
CD19		10	100.0
CD20		10	100.0
CD23		5	50.0
CD38		6	60.0
CD79b		10	100.0
CD103		1	10.0
CD123		2	20.0
FMC7		7	70.0
κ/λ	**K**	6	60.0
	λ	4	40.0

Table 6 represents a comparison between the immunophenotypic markers regarding MUM1/IRF4 among NHL group. It showed that no significant differences were found between the immunophenotypic markers regarding MUM1/IRF4 among NHL group.

Table 6:- Comparison between the immunophenotypic markers regarding MUM1/IRF4 among NHL group.

Marker	Present		Absent		P
	(N=10)	Mean±SD	(N=10)	Mean±SD	
CD23	5	29.6±6.7	5	3.5±3.9	0.451
CD38	6	2.9±3.4	4	12.0±1.4	0.395
FMC7	7	27.8±4.3	3	3.6±1.6	0.639
K	6	38.7±5.1	4	3.3±4.2	0.513
λ	4	28.7±3.9	6	11.5±7.1	0.410

^Independent t-test, *Significant

Table 7 shows that MUM1/IRF4 was significantly highest among multiple myeloma group (93.1±6.0) than among CLL group (24.2±11.6) and least among NHL (6.5±1.9) and among control group (0.6% ±0.5).

Table 7:- Comparison between Multiple myeloma, CLL, NHL and control groups regarding MUM1/IFR4.

Measures	Myeloma (N=20)	CLL (N=10)	NHL (N=10)	Control (N=20)	P
Mean±SD	93.1±6.0	24.2±11.6	6.5±1.9	0.6±0.5	**<0.001****
Range	75.7–99.9	0.1–69.1	0.5–39.4	0.1-1.5	
Homogenous groups	a	b	c	d	

^ANOVA t-test, **Highly significant. Homogenous groups had the same letter (a, b, c, d)

Discussion:-

In this study, flowcytometric immunophenotyping (IPT) was applied to detect the expression of MUM1/IRF4 in three different entities of B cell malignancies; multiple myeloma, CLL, and NHLs. The aim was to identify its significance as a diagnostic marker in those three entities, as a step for further studies on its significance in new therapeutic strategies.

In our study MUM1/IRF4 was significantly higher among the MM group than among the control group. These results were consistent with Heintel et al., 2008 [6] as they found that MUM1 gene was involved in the translocation t(6:14)(p25;q32) identified in multiple myeloma. This translocation leads to juxtaposition of MUM1 gene on chromosome 6 to the Ig heavy chain locus on chromosome 14. They also found that MUM1 was expressed in late plasma cell directed stages of B cell differentiation, suggesting that MUM1 may serve as a marker for neoplasms derived from these cells. $MUM1^+$ cells range from centrocytes to plasmablasts/ plasma cells. Therefore, MUM1 was considered to provide a marker to help in the identification of transition from $BCL\text{-}6^+$ (germinal center) B cells to $CD138^+$ immunoblasts and plasma cells.

Our results also agreed with Agnarelli et al., 2018. [7] They found that IRF4 plays an essential role in the genesis of MM, as it is the main regulator of the aberrant gene expression. They found that IRF4 was highly expressed in B cells and plasma cells; where it played important roles in controlling differentiation of B cells to plasma cells and immunoglobulin class switching. MUM1 was found to be over expressed in MM cells, they concluded that it was required for the survival of activating mutations and translocations involved in MM.

Another study done by Zhang et al., 2016 [8] acknowledged the same results. They reported that IRF4 promotes cell proliferation by c-Jun N-terminal kinases (JNK) pathway and that silencing IRF4 in myeloma cell lines may inhibit myeloma cells proliferation and it may as well induce myeloma cell apoptosis. These results demonstrated that IRF4 plays crucial roles in myeloma genesis and disease progression and it could be used in MM treatment through IRF4 inhibition.

Butrym et al., 2017 [9] added that Immunomodulatory drugs (derivatives of thalidomide in particular) are commonly used in treatment of MM and they are known to target a protein called cereblon (CRBN). They assessed the association of polymorphic IRF4 and CRBN alleles with MM susceptibility, prognosis and response to treatment.

They found that IRF4 allele was more frequently pronounced in patients than healthy individuals while CRBN allele was more pronounced in good responders to the treatment. These results highlight the prognostic significance of the IRF4 and CRBN polymorphisms in MM patients.

As for the relation between MUM1 and other IPT markers of MM (CD38, CD138, CD19, CD20, CD45, CD56, CD11b, κ/λ), our work revealed no significant correlation between them. But interestingly, κ was expressed in 18/20 (90%) of the MM patients also expressing MUM1 and though this did not yield a statistically significant result, yet it has been remarked. This may be attributed to the small sample size. While in a study by Jeong et al., 2012 [10] they did not favor using flowcytometry technique (FCM) to analyze immunoglobulin light chain restriction.

Our work could not establish a correlation between MUM1 and CD138 as both were expressed in 100% of cases. In contrast, Kawano et al., 2012 [11] highlighted that though CD138 is a hallmark of plasma cells and MM cells, yet decreased expression of CD138 was commonly found in plasma cells of myeloma patients. Further evaluation showed that low CD138 was commonly associated with down regulation of IRF4 and high expression of BCL6. These observations were indicative of an immature phenotype of MM with less sensitivity to lenalidomide, poor prognosis and worse survival. They suggested that this should be recognized as a new clinical entity and that establishment of a new therapy protocol for them is needed to improve their poor outcome.

In the current study, MUM1/IRF4 revealed a significantly perfect diagnostic performance and diagnostic characteristics in terms of MM diagnosis. The same was reported by Zhang et al., 2013. [12] They found out that IRF4 was a characteristic marker of myeloma cells in MM, and that IRF4 positive patients displayed advanced disease stage.

In our study MUM1/IRF4 was significantly higher among the CLL group than among the control group. These results were consistent with Ubieta et al., 2017. [13]

MUM1 has shown significantly moderate diagnostic performance and characteristics comparing CLL group to control group in our work. Brown, 2013 [14] reported similar results as well, and reported that there is a strong evidence that indicates IRF4 involvement in CLL. She claimed that IRF4 acts as a translocation breakpoint partner and in some cases, it also shows somatic mutations like point mutations, copy number variation or rearrangement. Meanwhile, how IRF4 aberrations promote CLL remains unexplained. [14]

In the current work, there was no significant correlation between the IPH markers assessed by flowcytometry regarding MUM1/IRF4 among CLL patients; which included CD5, CD19, CD20, CD38, CD23, FMC7, CD79b and κ/λ.

In our study, MUM1/IRF4 was significantly higher among the NHL group than among the control group. It had moderate diagnostic performance and characteristics among NHL patients. These results were consistent with Wang et al., 2014 [15], they found out that IRF4 over expression is the main feature of activated B-cell-like type of Diffuse Large B Cell Lymphoma (DLBCL), CLL and MM. They even added that it is also over expressed in almost 100% cases of Hodgkin lymphoma (cHL). They reported that IRF4 is associated with a different gene expression pattern in each type. Associated gene signatures include a pool of important genes involved in B cell development, oncogenesis, cell cycle regulation and cell death like BATF, LIMD1, CFLAR, PIM2 and CCND2 genes. These findings implicated IRF4 in these hematological malignancies. Suggesting that; in clinical practice; IRF4 may serve as an important prognostic and diagnostic marker for those previously mentioned hematological malignancies. [15]

In previous studies, MUM1 was specifically linked to certain entities of NHL, such as; DLBCL, and Mantle cell lymphoma (MCL). Lu et al., 2016 [16] have concluded the importance of linking IRF4 to DLBCL, which is the most common type of NHL in adults. While, Gualco et al., 2011 [17] have studied the value of MUM1 in MCL. They studied MUM1 as a marker of transition from BCL-6 positive (GC) B-cells to subsequent steps of B-cell maturation to plasma cells, so that MUM1 is expressed not only in post-GC cells but also in final stages of the intra-GC phase.

In the current work, there was no significant difference between the IPH markers assessed by flowcytometry regarding MUM1/IRF4 among NHL patients which includes CD 5, CD19, CD20, CD23, CD38, FMC7, CD79b, CD10, CD103, CD123 and κ/λ.

This study reveals that MUM1/IRF4 was significantly highest among multiple myeloma group than among CLL group and least among NHL group, which was also observed by Heintel et al., 2008. [6] They found high MUM1 expression in all 60 multiple myeloma patients involved in their study.

Uranishi et al., 2005 [19] also found that MUM1 was expressed in approximately 50% of B-CLL/SLL cases, MUM1 was also expressed in variable proportions in the different NHL entities; 73% of DLBCL and 20% of MZL, whereas it was not found in any cases of MCL or FL.

Conclusions:-

Under the limitation of this study, it can be concluded that identification of MUM1/IRF4 may play an important role in the diagnosis of B cell malignancies with a specifically significant role as a phenotypic marker in multiple myeloma.

Conflict of Interests:

The authors of this study declare no conflict of interests.

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