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Detection of oncoproteins in the Acute Promyelocytic Leukemia (APL) subtypes

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ABSTRACT

Acute promyelocytic leukemia is classified with a classic translocation t(15;17) (q22;q12), and the other subtypes such as: t(11;17) (q23;q21) , t(11;17)(q13;q21) t(5;17) (q35;q21). Unfortunately, about 5% of APL subtypes, do not have a good prognosis due to resistance to ATRA. Therefore, there is a major concern for hematologists and oncologists to diagnosis subtype of APL and start the treatments as soon as possible. 28 patients with suspected acute promyelocytic leukemia who hospitalized in two Iranian general hospitals were under study . In this study, we applied molecular and western blotting methods to detect major oncoproteins in APL subtypes. Cytogenetic results have been shown that the PML / RARa oncogene is positive in 26 of patients, and two of them did not have appreciated result. The results of western blotting showed that in 26 patients, the proteins with molecular weights 115 and 85 KD was detected by specific anti-RARa antibodies. The western blotting assay for one patient showed the protein with molecular weight of 85 KD was detected by specific anti- PLZF antibodies .The results indicate that the molecular and western blotting techniques were able to differentiate the APL with t (15; 17) from the other subtypes, like t (11; 17). We indicated by western blotting, two oncoproteins: PML / RARa and PLZF / RARa, respectively to the relative two translocation t (15; 17) and t (11; 17). Western blotting techniques can be a substantial contribution of to the final diagnosis of leukemia.

Keywords: Acute promyelocytic leukemia, oncoprotein, western blotting

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a subtype of acute leukemia which characterized with a rapid onset blood myeloid cell proliferation and aggressive clinical features including weakness, bone pain, anemia and DIC[1]. APL with morphological characterization is classified in two subtypes: c-APL or hypergranular and v-APL or hypogranular [2,3]. APL is also classified (WHO classification) with a classic translocation , t(15;17) (q22;q12), and at least 7 subtypes t(11;17 ‹)(q23;q21) , t(11;17)(q13;q21) t(5;17) (q35;q21), inv (17) ‹t(4:17) (q12;q21)‹ t(x;17) (p11;q21) (3- 5). Almost 100% of APL patients carry RARa (the retinoic acid receptor alpha) gene on chromosome 17 which fused to its partner oncogenes on different chromosomes [6]. About 97% of APL with t(15; 17)(q22; q12) , RARa is joined the PML gene on chromosome 15. In 3% of APL with other translocation, the RARa gene on chromosome 17, joined to PLZF, NPM, NUMA, STAT5b, PRKAR1A, BCOR, FIP1L1 genes on the respective chromosomes [6-9]. Recently some patients with APL have been reported with a chimera translocation such as t (5; 17; 15) (q35; q21; q22) and the t (12; 15; 17) (q24; q24; q11), [9, 10]. Although the APL with difference translocation usually are treated with ATRA in combination with other chemotherapy drugs and has a good prognosis by comparison with other AML, but the APL with oncogene PLZF-RARa is resistant to ATRA. Due to the PLZF-RARa rearrangement which is the second most common rearrangement in APL and ATRA –resistant, therefore it is necessary to identify PLZF-RARa oncoprotein to monitor the treatment [11,12].

MATERIALS AND METHOD

Sample preparation:

Anti coagulated peripheral blood and Bone marrows were obtained from 28 patients with APL, who were admitted to oncology clinic of two Iranian general hospitals (Shariati and Taleghani) in Tehran. Laboratory tests such as CBC, BM, cytochmistry and Immunophenotyping were assayed in hospitals. RT-PCR assay was employed to detect cytogenetic abnormality of patient's leukemic cells.10 healthy voluntary adult and HL60 cell line were used as the negative controls.

Cell Culture:

HL60 cell were obtained from Stem Cell Research Center (Shariati Hospital), the characterization of HL60 was detected in Stem Cell Research Center. Cells cultured in RPMI medium and passage frequent time until the number of cells reached up to 80% of the first passages cell number.

Promyelocytes isolation:

Mononuclear cells (dominantly promyelocytes) of patients were isolated by ficoll and were

washed immediately by phosphate buffer and counted using hemocytometer chamber. The washed cells were diluted with FBS containing 10% DMSO by 8:10 and froze at -20 °c. Mononuclear cells of patients and WBC healthy control and HL60 cells defrosted and laysed using a modification of the method described by Realson and colleagues (13), briefly patients and control isolated cells added in 100 ml of lysed buffer (Tris 40 mmol / l, EDTA 1mmol / l, Urea 7 mol / l, Thiourea 2 mol / L, DNase 0.1mg/ml, RNase 0.1mg/ml, COCKTAIL 1%, DTT 1%, CHAPS 4%). After vortexing the sample vigorously, the mixture centrifuged using refrigerated centrifuge at 20,000 g for 30 min. The upper layer later was collected and concentrated using 0.5 µ filter (Milipore Co). The cell lysate concentrated of patients and controls were assayed, according to the Bradford method using a protein measurement kit (Bio-Rad), the final concentration was 1200µg /ml .

Electrophoresis samples preparation:

The concentrated cell lysate was diluted 3: 1 with sample buffer (DTT: 4ml, SDS10%: 0.3gr, separating gel buffer:1.6 ml, Glycerol 87% :2.5 gr, DW: 20 ml, Bromophenol Blue, 0.5 mg) and boiled for 3 minutes.

Analysis of APL oncoproteins Specificity by Western blotting:

The Western blotting protocols were modified from those described by Blot (14) and Towbin (15). 40 µl of the prepared cell Lysate of patients, healthy control , HL60 cell lines and 10 µl of positive control (peptide PLZF (Novusbiological) and 5 µl of protein ladder (Governor) was added the 10% polyacrylamide gel and run at 120 V for 2 hours in the running buffer (0.3% tries, 1.44% Lysine, 1% SDS). The protein was transferred from gel to a 0.45 Nm, nitrocellulose membrane (Sartorius Co) and run at 100 V for 90 min in blotting buffer. Nitrocellulose membrane containing the separated samples and control proteins were blocked with 5% fat-free milk in TBS for 30 min at room temperature. After the membrane was washed for 4 times with washing buffer (TWEEN-20: 5gr in 100ml PBS), they were incubated overnight with Rabbit-Anti-RARa-Antibody and Anti-PLZF antibody (Abcam) diluted 1:100. The membrane was washed as above and incubated for 90 min with Goat anti-Rabbit conjugated (HRP) (Abcam) diluted 1:10000. After it was washed 4 times with washing buffer, the membrane was incubated for 1 hour with D-amino-Benzidine solution (NaCl: 0.9mg, Tris Hcl: 1.57mg, DAB: 0.072mg, DW: 100ml) adjusted PH to 7.4. The proteins were highlighted in brown color.

RESULTS AND DISCUSSION

Morphology results showed that in PB and BM of all patients, the majority of cells were blast and promyelocytes with rough azorophilic granules. The cytochmistry assay by Sudan Black B (SBB)

showed that scoring were (1-4+) in all patients, figure 1 shows the Gimsa and cytochmistry staining in one APL patients .

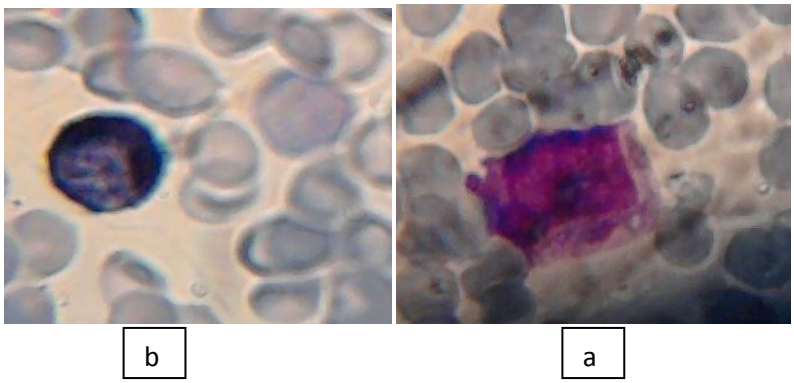

Figure 1: Peripheral Blood Smear of a patient with APL (PLZF/RARa oncoprotein positive): a; a promyelocyte with Auer rod, b; SBB strong positive in a PB of patient

The Immuno phenotyping results showed that all of 28 patients in terms of number of reviews are positive for CD13 CD33 CD117 by flow cytometry. Cytogenetic analysis results showed that, in 26 patients PML / RARA oncogene were positive and in 2 others were negative.

Western blotting results:

40µg protein of each patient's samples and controls were analyzed by western blotting. The sample of all 28 patients and controls were characterized, because of that the western blotting results of 28 patients represent the same antibody (Anti-Rare antibody) results, so characterization results from only few of samples are presented in Figures 2-4 .

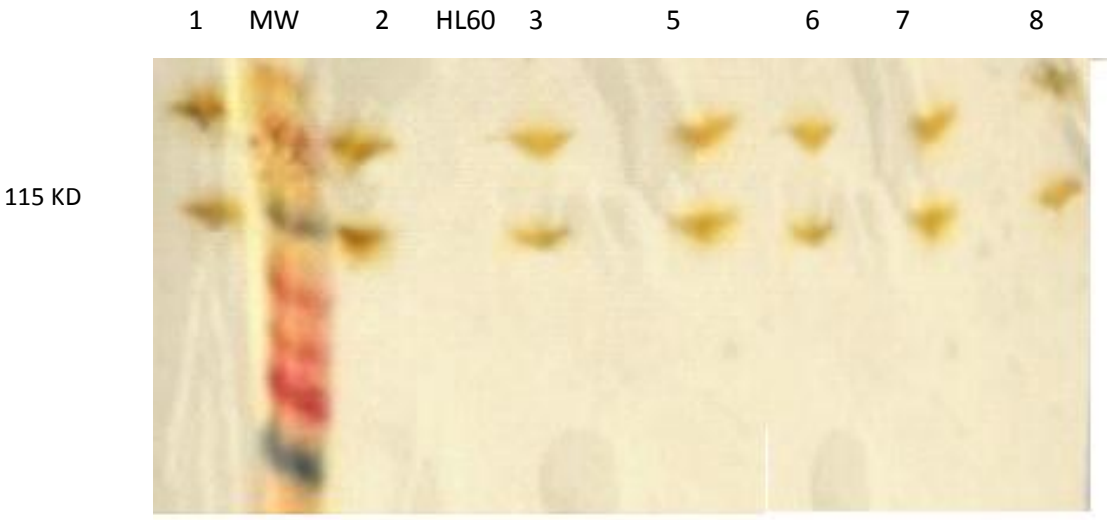

Figure 2: The results of Western blotting of samples No (1,2,3,5,6,7 and 8) with anti-Rare antibodies. The results indicate that there are two bands with molecular weights of 115 and 85 KD. HL60 cell Lysate as a negative control lacking the protein bands.

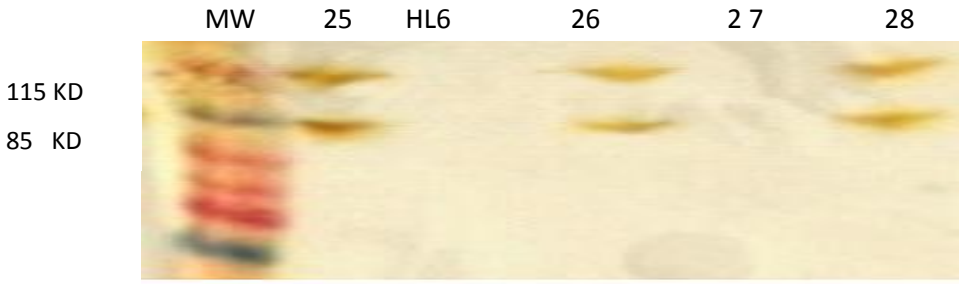

Figure 3: The results of Western blotting with anti-Rare antibody .Samples (25, 26 and 28). The results indicate the presence of two bands with molecular weights of 115 and 85 KD. HL60 cell Lysate as a negative control and sample 27 lacking the protein bands.

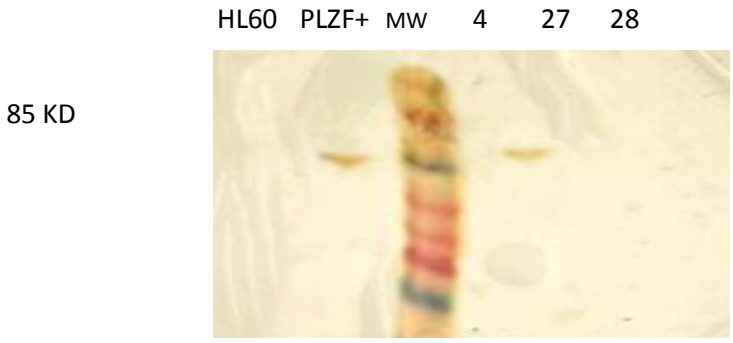

Figure 4: The results of Western blotting with anti-PLZF antibody indicate that there is a band with a molecular weight of 85 KD in sample No. 4, Positive control (PLZF peptide). HL60 cell and samples No 27 and 28 do not show any band in the gel.

Acute Promyelocytic Leukemia (APL) results from a reciprocal translocation that fuses the oncogene such as PML, PLZF, NPM, NUMA tumor suppressor to that encoding the retinoic acid receptor alpha (RARa) (5). The blood cell morphology in PB and BM of all patients slides, indicated that, the majority of cells were blast and promyelocyte . It would be explained that, there is a relationship between the number of BM-Blast and PB- blast ($r = 0.962$, $p < 0.001$). Although the CBC and cell morphological detection in PB or BM smears is the first step of laboratory diagnosis to determine APL [3], in 5-10 % of patients with APL are not detected by morphological and cytochemical methods [16]. Therefore, we need to use molecular technique for detecting APL. In our study, we have seen the relation between results of cytogenetic analysis and immunophenotyping with a statistical coefficient ($r = 0.96$, $p < 0.001$) in 26 of patients. Based on the data from this study using western blotting by mouse antibody against RARa, the protein with molecular weight 115KD was detected. However, another protein with a molecular weight of 85 KD was also identified in this study. To eliminate the possibility of error in the separation and break down protein with 115 KD and thereby unrealistic protein 85 KD, we used the western

blotting with the fresh sample in gel accompany high concentration of anti- protease and low concentration of DDT . The results showed the same as results that we have reported above. In a report presented by John V Raelson a protein with 85 kDa band could be induced by treatment with ATRA and creating this piece is to break the protein [14]. In this review, 10 healthy controls along with HL60 cell line were used as a negative control. Proteome of HL60 cell has lack of APL/RARa and PLZF/ RARa oncoproteins, so it gave us the opportunity to make use of HL60 as a negative control [17]. Only protein lysate of patient No 4 who was lacking the t(15;17), reacted by anti PLZF antibodies. The PLZF-peptides were used as the positive control in this study. This protein has a molecular weight of 85KD which is consistent with the results of other studies [13, 18.] Fabien Guide and collogues used the mice blast which transfected with PLZF-RARa oncogene, they find out, the proteins with molecular weights of 35 , 70 and 105 KD by Western blotting. However, it seems that cells were used in this studies were not relevant to human samples[19]. However, we were able to identify the oncoprotein without gene transfer of translocation in human samples outside the cell by Western blotting technique. Statistical analysis of this study indicates that there is a relation between the results obtained from patient samples by Western blotting and cytogenetic, ($r = 0.962$ and $P < 0.001$). Patients who have the t (15; 17), the oncoprotein PML / RARa with molecular weight of 115 KD was positive. In this study, we research over 90% of patients who were studied, had a PML-RARa protein. EHA-dekking and colleagues reported that in 92% of patients with classical APL was identified the PML-RARa oncoprotein and about 5% of them detected non-classical type of oncoproteins [5]. Due to low concentration of protein lysate of sample No 27, we do not recognized any band in western blotting assay, in spite of the results which came out of morphological and cytochmistry tests suggested as APL diagnosis . However, despite its exposure APL with the t (11; 17) and t (15, 17) in a group of leukemia called APL, joining the two separated research level course are distinguished from each other [20]. In the end we would like to suggest that, because of the patients with $t^{11;17}$ are poor response to ATRA it is important to detect the APL subtypes and treat with appropriate therapy regime like A2S3O [21,22] and the Ubiquitin-specific peptidase 37 (USP37) very soon [23].

CONCLUSION:

The Western blot test is a useful method to detect proteins; we recommend the use of this method for detecting oncoprotein in leukemia as a molecular diagnosis. This method have two advantages: first it is a high specificity test because of specific binding of the antibody against oncoprotein, and the other advantage is that the time required to perform this method is less than cytogenetic assay.

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