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Hsp70, in Combination with IL-15 and PD-1 Blocker, Interferes with The Induction of Cytotoxic NK Cells in Relapsed Acute Myeloid Leukemia Patients

Javad Firouzi, Ph.D.[1, 2, 3], Abbas Hajifathali, M.D.[4], Masoumeh Azimi, M.Sc.[2], Neda Parvini, M.Sc.[5], Fatemeh Ghaemi, M.Sc.[2], Niloufar Shayan Asl, M.Sc.[2], Amir Abbas Hedayati Asl, M.D.[2], Majid Safa, Ph.D.[1, 3, 6*], Marzieh Ebrahimi, Ph.D.[2, 7*]

1. Department of Tissue Engineering and Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
2. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
3. Cellular and Molecular Research Centre, Iran University of Medical Sciences, Tehran, Iran
4. Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
5. Cellular and Molecular Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Kurdistan, Iran
6. Department of Hematology and Blood Banking, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
7. Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Abstract

Objective: Natural killer (NK) cells are critical immune cells for acute myeloid leukemia (AML) targeting. However, little is known about the relationship between using checkpoint inhibitors and heat shock protein 70 (Hsp70) as NK cell activators to control AML. Therefore, the study aims to find the best formulation of Hsp70, human PD-1 (Programmed cell death protein 1) blocker, and interleukin 15 (IL-15) to activate NK cells against AML.

Materials and Methods: In this experimental study, the NK cells were isolated from mononuclear cells (MNCs) by using magnetic activation cell sorting (MACS) and were activated using the different combinations of Hsp70, PD-1 blocker, and IL-15 and then followed by immunophenotyping, functional assays to estimate their killing potential, and evaluation of expression pattern of *PRF1, PIK3CB, PD-1, AKT-1, FAS-L, TRAIL,* and *GER A* and *B.*

Results: The expression of PD-1 was significantly (P<0.05) reduced after NK cell activation by the different formulas of IL-15, Hsp70, and PD-1 blocker. The expression of NKG2A in the treated NK cells was reduced particularly in the IL-15 (P<0.01) and IL-15+PD-1 blocker (P<0.05) groups. The addition of Hsp70 increased its expression. The cytotoxic effect of NK cells increased in all groups, especially in IL-15+PD-1 blocker besides increasing interferon-gamma (IFN-γ), Granzymes, and perforin expression (P<0.05). All IL-15+PD-1 blocker group changes were associated with the up-regulation of *PIK3CB* and *AKT-1* as key factors of NK cell activation. The presence of Hsp70 reduced IFN-γ releasing, and down-regulation of *PIK3CB, AKT-1*, Granzymes, and *Perforin* (P<0.05).

Conclusion: We suggested the combination of IL-15 and PD-1 blocker could enhance the killing potential of AML-NK cells. Moreover, Hsp70 in combination with IL-15 and PD-1 blocker interferes activation of AML-NK cells through unknown mechanisms.

Keywords: Acute Myeloid Leukemia, Hsp70, Immunotherapy, Natural Killer Cells, PD-1

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of disorders characterized by malignant clonal proliferation of myeloid blast cells in the bone marrow (BM) and peripheral blood, leading to cytopenia, infections, and bleeding (1, 2). Although many therapeutic interventions have been explored to treat patients with AML, chemotherapeutic regimens remain a crucial therapy component for these patients (3). In the AML treatment chemotherapy guideline, the two antineoplastic agents, fludarabine and busulfan, are well-known. Fludarabine shows promising results in treating relapsed/refractory patients with AML; however, the neurotoxicity effect of this agent limits the use of high-dose fludarabine (4).

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***Corresponding Addresses: P.O.Box: 1449614535, Department of Hematology and Blood Banking, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran**
P.O.Box: 16635-148, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
Emails: safa.m@iums.ac.ir, m.ebrahimi@royan-rc.ac.ir

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Despite its lower costs and acceptable efficiency over the decades, the significant side effects of busulfan, including mucositis of grade 2 or higher, led to the displacement of this agent with more expensive but secure monoclonal antibody imatinib (5).

To date, natural killer (NK) cell-based immunotherapy is one of the most current innovative immunotherapeutic techniques, unleashing the immunological suppression of NK cells to attack a variety of malignancies (6). In AML, dysfunctional NK cells or immunosuppressive features of AML cells and their prognostic relevance justify using NK cell-based immunotherapy to restore impaired NK cell cytotoxicity against AML (7). Meanwhile, treatment with busulfan and fludarabine inactive NK cells (PMID: 2933271) with unknown mechanisms. Another defined mechanism that makes weak NK cells, as well as T cells in patients with AML, is the overexpression of inhibitory immune checkpoint molecules such as programmed cell death ligand-1 (PD-L1) and PD-L2 up-regulated in blasts (8). Furthermore, heat shock protein 70 (Hsp70) is an essential component of the protein folding system called chaperones and protects the cells from stress-induced damage (5).

Hsp70 as an antigenic peptide (9) can be used as a tumor-specific vaccine (10). Furthermore, Hsp70 induces the release of pro-inflammatory cytokines from innate immune cells, increasing the expression of costimulatory molecules (11). In addition, Hsp70 activates the NK cell cytotoxic effects against the Hsp70 representing tumor cells (12). Despite promising outcomes resulting from investigations performed on PD-1 blocker base immunotherapy on solid tumors, the efficacy of this approach is not studied on hematologic malignancies. Therefore, in the present study, we assessed the combined effect of PD-1 blocker and Hsp70 on the activation of NK cells derived from patients with relapsed AML under treatment of Busulfan and fludarabine.

Materials and Methods

Patients

Nine patients diagnosed with relapsed non-M3 AML were treated with fludarabine and busulfan at the Blood and bone Marrow Transplantation center in Taleqani hospital (Tehran, Iran) between 2019 and 2022 and were joined in this experimental study. All patients signed informed consent before entering the study, and the advantage of their admission was explained verbally and in writing. All procedures in the present study were performed following the relevant guidelines and regulations of the Royan Institute and approved by the Institutional Review Board and Ethics Committee of The Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC.1400.055).

Cell culture and reagents

MNCs were collected from Peripheral blood via Ficoll-Hypaque (inno-train, 108 Utica Street Clinton NY 13323, USA, Cat No: 002041600) concentration gradient centrifugation. The Ficoll-Paque centrifugation is done as per the manufacturer's protocol. The KG-1 cell line was purchased from Royan Institute Cell Bank (Tehran, Iran, CCL-246). KG-1 cells were cultured in RPMI-1640 containing 10% FBS (GIBCO, Cat. No: 26140-079), 3 mM L-glutamine (GIBCO, Cat. No: 25030-024), 1% Pen/Strep antibiotics (GIBCO, Cat. No: 15070-063), and a humidified atmosphere of 5% CO_2 at 37˚C.

Immunophenotyping of cells

Peripheral blood mononuclear cells (PBMCs) derived from Patients with AML, were stained with Anti-human CD335-FITC (NKP46; Cat. No: 331922), Anti-human CD159a-PE (NKG2A; Cat. No: 142803), anti-human CD337-PerCp/Cyanine5.5 (NKP30; Cat.No:325216), anti-human CD314-PE (NKG2D; Cat. No: 320806), and BD Simulates CD3-FITC/CD16+CD56-PE (Cat. No: 342403) to evaluate the abundance, phenotype and function of NK cells by BD FACS Calibur flow cytometer instrument (BD Biosciences, San Jose, California, USA) and analyzed in FlowJo software Ver.10.6.1. All fluorescence-labelled antibodies were acquired from Biolegend (USA).

NK cell isolation, cultivation and cytotoxicity assay

NK cells were isolated from MNCs of patients with AML before the initiation of induction chemotherapy (n=9) by selecting $CD56^+$ cells with magnetic-bead separation (Miltenyi Biotech, USA). The isolated cell purity was calculated and confirmed by flow cytometry and specific antibody against $CD56^+$. Then, NK cells were divided into five groups (1×10^4 cells/mm^2) [NK cells without any factor, NK cells that received Human IL-15 (10 ng/ml), NK cells that received IL-15 (10 ng/ml) and PD-1 blocker (0.5 µg/ml), NK cells received IL-15 (10 ng/ml) and Hsp70 (20 µg/ml), and the last group of NK cells received IL-15 (10 ng/ml), PD-1 blocker (0.5 µg/ml) and Hsp70 (20 µg/ml)]. These NK cell groups from patients with AML were assessed for their capacity to kill NK cell-sensitive KG1 cells. The KG-1 cell line was inactivated using Mitomycin C (20 µg/ml; 2×10^6 cells per well) to impede their proliferation, subsequently, Calcein AM was used to label them (Incubation for 45 minutes at 37˚C with 5% CO_2). The labelled cells were washed with phosphate-buffered saline without calcium and magnesium (PBS^-, Gibco, Cat. No: 21600-051, USA) and resuspended in X-X IVO medium (Lonza, BE02-060Q, Belgium) with 10% FBS. KG1 cells were co-cultured at an effector: target (E: T) ratio of 10:1, which incubated for 24 hours at 37˚C in 5% CO_2. Cytotoxicity level was evaluated with calcein/propidium iodide (PI) staining by flow cytometry technique. Tumor cells were grown in the same media without NK cells exposure considered as control groups.

IFN-γ production and LDH release evaluation

Human lactate dehydrogenase (LDH) and Interferon-gamma (IFN-γ) ELISA Kits (MyBioSource, Cat.No: MBS009535 and MABTECH, Cat. No: 3420-1 HP-1) were used to test lactate dehydrogenase (LDH) release from the

KG1 cell line, and IFN-γ production by NK cells, following the manufacturer's procedure. Briefly, 100 µL per well of standard solutions or samples was divided into aliquots in duplicate into a precoated 96-well plate. After discarding the plate content, 100 µL/well of biotinylated anti-human LDH and IFN-γ antibodies were added to each well. After rinsing three times with PBS, a prepared avidin-biotin-peroxidase complex (ABC) working solution (100 µL/well) was added to each well. Finally, after washing with PBS^-, a prepared stop solution of 100 µL/well was added to each well, and the plate was read at 450 nm in a microplate reader.

RNA isolation and qRT-PCR

Identic to the manufacturer's procedure, whole cellular RNA (1 µg) was extracted from cells using TRIzol reagent, and then assessed the quantity and quality of RNA samples on Nanodrop and gel electrophoresis. Only RNA samples with RNA Integrity Numbers (RIN) > 6 were included in the analyses. Then reverse transcriptional reaction was conducted to obtain cDNA by Prime Script RT Master Mix. According to the manufacturer's instructions, 1 µg of total RNA was used to synthesize cDNA by an RT-for-PCR kit (Takara Bio, Inc., Otsu, Japan). Primers (*PRF1*, *PIK3B*, *PD-1*, *AKT-1*, *FAS-L*, *TRAIL*, *GERA* and *B*) were designed and certified using NCBI-Primer BLAST (Table 1). Specimens were duplicated from three independent trials; β2-microglobulin RNA levels were employed as an internal reference for all experiments. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ methods.

Statistical analysis

The data were presented as mean ± standard error of mean (SEM), and the statistical analysis was performed using GraphPad Prism software (version 9, University of California San Diego, USA). The Wilcoxon rank test or Mann-Whitney tests examined the statistical significance of the two groups and kruskal wallis test was used when comparing more than two groups. $P<0.05$ were considered statistically significant.

Results

The main biological characteristics of the patients are in Table 2.

The expression pattern of PD-1 on NK, NKT, and T cells in non-M3 relapsed AML

Autologous NK cell therapy is accessible for cancer immunotherapy. However, the main question is whether these NK cells have enough ability to overcome cancer cells. Therefore, we evaluated the expression pattern of PD-1 as an inhibitor marker on NK cells in MNC derived from whole blood samples of seven patients with AML who were treated with Fludarabine and Busulfan. Immunophenotyping of the whole blood of patients determined that 63.25 ± 5.3% of total lymphocytes were T cells, of which 15.93 ± 2.76% were PD-1 positive. Instead, NK (CD56+/16+CD3-) cells were about 12.3 ± 4.18% of total lymphocytes with 7.05 ± 1.14% expression of PD-1, and NKT (CD56+/16+CD3+) cells were 6.02 ± 2.68% of total lymphocyte with 11.47 ± 3.28% PD-1 expressing cell (Fig.1).

Table 1: Primer sequences used for reverse transcription-quantitative polymerase chain reaction

Gene name	Primer sequence (5′-3′) (10-50 bp)	Length (bp)	Temperature (˚C)	Absorbance (OD)
FasL	F: TGCCTTGGTAGGATTGGGC	19	58.83	2-4
	R: GCTGGTAGACTCTCGGAGTTC	21	61.78	2-4
TRAIL	F: TGCGTGCTGATCGTGATCTTC	21	59.82	2-4
	R: GCTCGTTGGTAAAGTACACGTA	22	58.39	2-4
PDCD1	F: CCAGGATGGTTCTTAGACTCCC	22	62.12	2-4
	R: TTTAGCACGAAGCTCTCCGAT	21	57.87	2-4
PIK3CB	F: AGAGCACTTGGTAATCGGAGG	21	59.82	2-4
	R: CTTCCCCGGCAGTATGCTTC	20	61.40	2-4
AKT1	F: AGCGACGTGGCTATTGTGAAG	21	59.82	2-4
	R: GCCATCATTCTTGAGGAGGAAGT	23	60.65	2-4
PRF1	F: GGCTGGACGTGACTCCTAAG	20	61.40	2-4
	R: CTGGGTGGAGGCGTTGAAG	19	60.98	2-4
GZMA	F: CAGCAGCCACAATGAGGAAC	20	59.35	2-4
	R: TGCAGTCAACACCCAGTCTT	20	57.30	2-4
GZMB	F: TACCATTGAGTTGTGCGTGGG	21	59.82	2-4
	R: GCCATTGTTTCGTCCATAGGAGA	23	60.65	2-4

Table 2: Main biological characteristic of Non-M3 relapsed AML patients

Variable	Number (%)
Age (Y)	
<50	10 (50)
>50	10 (50)
Sex	
Male	14 (70)
Female	6 (30%)
Missing data	11 (55)
Missing from RT-PCR analysis	2 (10)
Total	20 (100)

AML; Acute myeloid leukemia and qRT-PCR; Quantitative reverse transcription-polymerase chain reaction.

NK cells activation by IL-15, Hsp70 and PD-1 blocker

To assess the effect of IL-15, Hsp70, and PD-1 blocker as activation mediators for NK cell therapy, CD56+ cells were isolated from nine patients with non-M3 relapsed AML using manual MACS. 80.22 ± 2.54% of purified cells were NK (CD56+/16+CD3-) cells, 16 ± 1.85% were NKT (CD56+/16+CD3+) cells and lower than 1.82 ± 0.25% of them were T cells (CD56-/16-CD3+) (Fig.2A).

In the next step, purified cells were incubated with different components, including IL-15, PD-1 blocker, and Hsp70, which were reported to be essential for their expansion and activation (Fig.2B). The results displayed that the number of PD-1-expressing NK cells and the mRNA level of PD-1 was significantly reduced in all groups treated with IL-15 in combination with Hsp70 and PD-1 blocker, compared to inactive and IL-15 treated group ($P<0.05$, Fig.3A). Meanwhile, the expression of NKG2A, as an inhibitory receptor, was reduced in those groups that received in PD-1 blockers ($P<0.05$, Fig.3B). The results indicated that Hsp70 in combination with IL-15 can promote the expression of NKG2A ($P<0.001$, Fig.3B). Looking at NKP30 and NKP46 as activator receptors signified that their level reduced in those groups that activated with PD-1 blocker, Hsp70, and their combinations compared to inactive NK cells and IL-15 treated group ($P<0.001$ and $P<0.01$, Fig.3C, D).

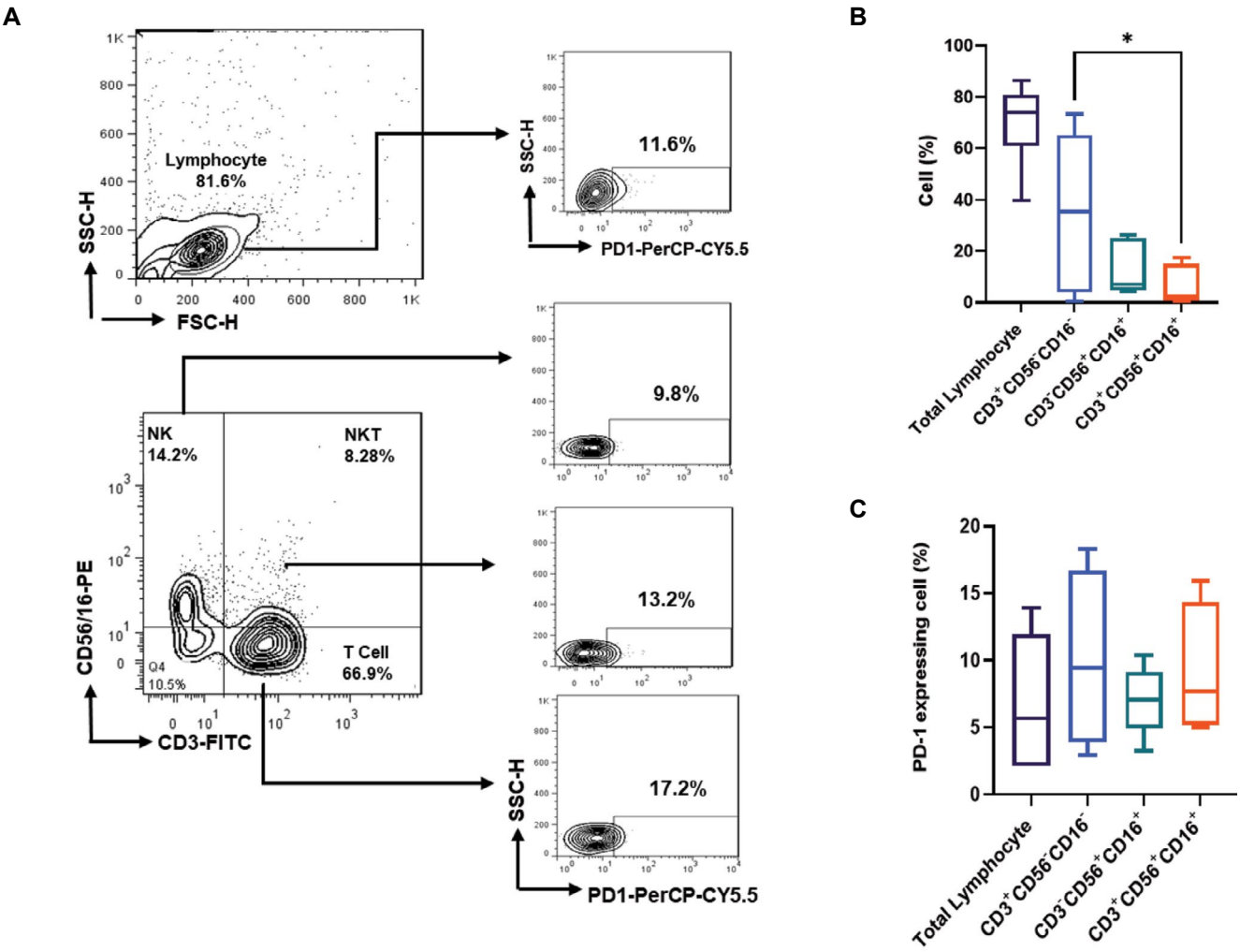

Fig.1: The PD-1 expression on T, NKT and NK cells derived from MNC of non-M3 relapsed AML patients. **A.** Schematic presentation and **B.** Quantification of different cell types and characterization of PD-1 expressing cell in patients. **C.** The PD-1 is expressed on 11.6% of total lymphocyte and 9.8% of CD56+ and CD16+ cells. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis was performed using unpaired two-way Anova (n=7). *; P<0.05, CD; A cluster of differentiation, MNCs; Mononuclear cells, PD-1; Programmed cell death protein, AML; Acute myeloid leukemia, and NKT; Natural killer T cells.

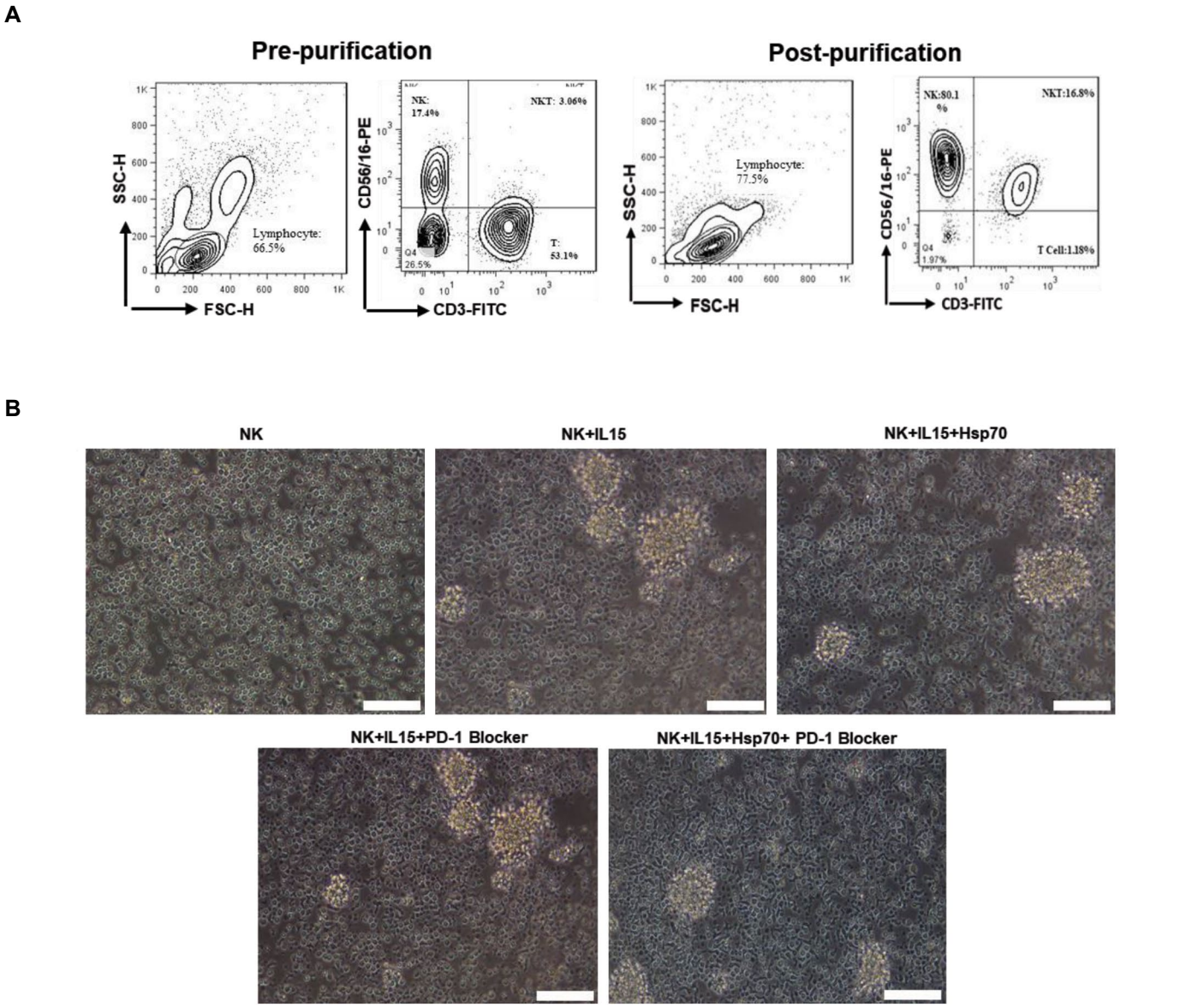

Fig.2: NK cell isolation and activation by using different components. **A.** The percentage of NK cells pre-purification was about 17% which riched to 80% with less than 2% T and 16% NKT cells post purification with MACS. **B.** Morphologically, NK cells in different activating components, including IL-15, Hsp70, and PD-1 blocker, displayed round clonies with the ability of expansion to the single activated NK cells (n=7). Images were obtained with a 20x objective, scale bar: 100 µm. NKT; Natural killer T cells, MACS; Magnetic activated cell sorting, IL; Interleukin, Hsp70; Heat shock protein 70, and PD-1; Programmed cell death protein 1.

The different activator combinations' effect on NK cell-mediated cytotoxicity

As mentioned earlier, the combination of IL-15, Hsp70, and PD-1 blocker reduced the expression pattern of both activating and inhibitory receptors of the patient's NK cells. Nevertheless, the main question is how these components affect the cytotoxic potential of NK cells. Our results indicated that although the cytotoxicity of treated NK cells on the KG1 cell line, as target cells, enhanced in all treated groups, it was dominant in groups with PD-1 blocker in their formulation (Fig.4A, B). Meanwhile, the rate of LDH released from KG-1 cells co-cultured with NK cells only exhibited elevation in IL-15+Hsp70+PD-1 blocker group that was not significant (Fig.4C). As the lactate dehydrogenase (LDH) assay is used in NK cell cytotoxicity assessment against tumor cells, its release is detected at 4 hours post activation (13). For the LDH release assay, we removed condition media after 24 hours, but our result did not show a significant difference between groups that may be associated with the NK and KG1 cells co-culture time which incubated over 4 hours. IFN-γ is another factor that is released and enhanced post-activation of NK cells. IFN-γ level significantly increased in IL-15+PD-1 blocker-treated group compared with inactive NK cells ($P<0.03$, Fig.4D). Also, we found that in those groups that Hsp70 was in their formulation, the secretion of IFN-γ significantly reduced (Fig.4D). The reason for this variation may back to use of fludarabine in these patients. Actually, it has been reported that fludarabine increases the secretion of interferon, and therefore, after using the Hsp70, its secretion no longer shows an increase (14).

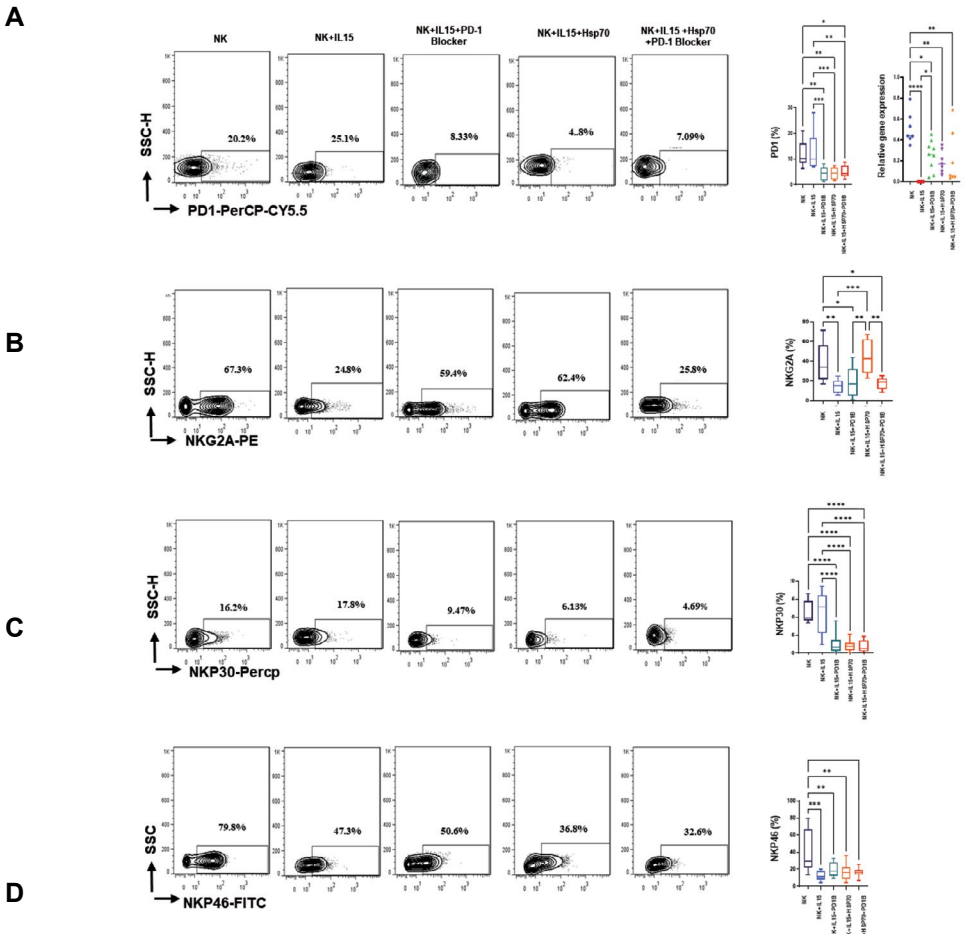

Fig.3: The expression of activating and inhibitory receptors in different actived NK cells. Purified NK cells were activated in presence of IL-15, Hsp70 and PD-1 blocker for 24 hours **A.** The expression of PD-1 was assessed at mRNA and protein levels. The results indicated of higher reduction of PD-1 in those groups that received Hsp70, PD-1 blocker and their combination than IL-15 or in inactive NK cells, (n=9, P<0.02). **B.** The expression of NKG2A was significantly reduced in all groups (n=9, P<0.04) except in group that received both IL-15 and Hsp70 (n=9, P>0.05). **C.** The expression of NKP30, and **D.** NKP46 as activator receptors reduced in all combinatorial groups post-activation (n=9, P<0.006), which means that their expression was higher in inactivated NK cells. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis was performed using unpaired two-way Anova. *; P<0.05, **; P<0.01, ***; P<0.001, ****; P<0.0001, NK; Natural killer cell, IL; Interleukin, Hsp70; Heat shock protein 70, and PD-1; Programmed cell death protein 1.

Expression of granzyme A and B in different formulations of NK activators

Granzymes are derived from serine proteases and expressed in cytotoxic T cells and NK cells (15). *GZMA* and *GZMB* are carried out and stored in the particular granules of resting NK cells and released after activation (16). Therefore, we assessed the expression of *GZMA* and *GZMB* in different groups of activated NK cells from seven patients with AML. The results indicated that IL-15 + Hsp70 + PD-1 blocker group upregulated the expression of both *GZMA* and *GZMB* (P<0.01, Fig.4E, F). Moreover, the lytic granules of NK cells contain the pore-forming protein perforin that, after the formation of immune synapses, creates a pore in the membrane of the target cell; finally, granzymes may enter the target cell cytoplasm and cleave several substrates, leading to apoptosis via the intrinsic pathway (17). Our result also showed that the expression of *PRF-1* in NK cells exposed to IL-15+Hsp70+PD-1 blocker was increased compared to all other groups (Fig.4G).

Fas ligand and *TRAIL* as death ligands on the surface of NK cells can activate target killing by attaching death receptors on the target cell, which activates NK cell cytotoxicity through caspase-8 dependent pathway extrinsic apoptosis (18). We expected that *Fas* ligand expression upregulated in activated groups. However, it was over-activated when the NK cells were treated with IL-15+Hsp70 (P<0.0001, Fig.4H). Similarly, *TRAIL* expression also increased in the IL-15+Hsp70 group but it was just significant compared to IL-15+PD-1 blocker+Hsp70 group (P<0.0089, Fig.4I). Studies showed that PI3K–AKT–mTOR pathway is the main pathway in regulating the development, differentiation, and activation processes of immune cells like NK cells (19). Also, *PIK3CB*, as a subunit of (phosphoinositide 3-kinase) *PI3K*, has a crucial role in NK cell cytotoxicity (20). In the assessment of the *AKT-1* gene, it seems that the PD-1 blocker in combination of IL-15 has the potential to increase its expression levels (Fig.4J). Also, the increased expression level of *PIK3CB* in the IL-15 + PD-1 blocker-treated NK cells was significant in comparison to other groups (P<0.01, Fig.4K) excluding the IL-15+PD-1 blocker+Hsp70 group which was not significant (P>0.05, Fig.4K).

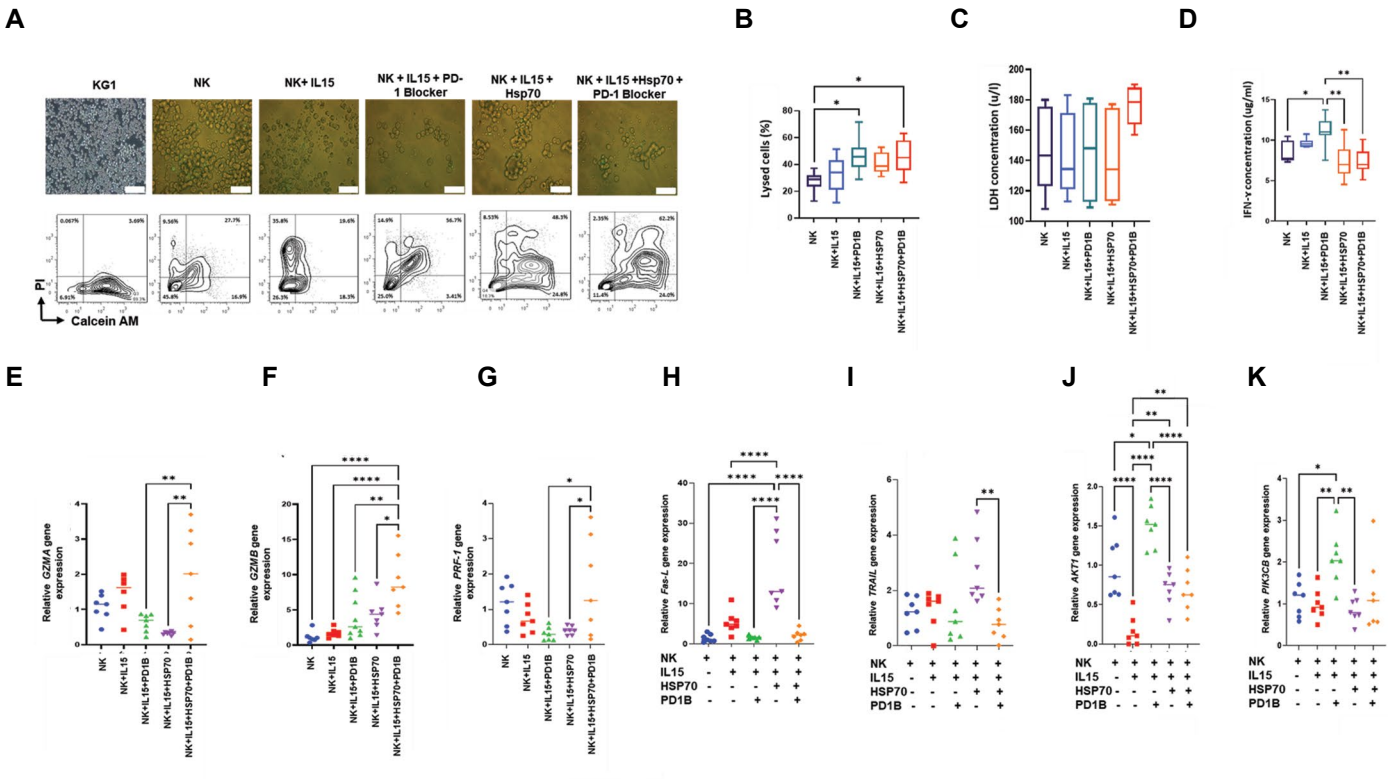

Fig.4: The effect of different activator combinations on NK cell-mediated cytotoxicity. **A.** Morphological illustration and flowcytometry results of different activator combinations' effect on NK cell-mediated cytotoxicity against KG-1 cell line. line. Images were obtained with a 40x magnification, scale bare: 100 µm for KG1 cell line, 50 µm for KG1 co-cultured with NK cells. **B.** NK cell cytotoxicity potential increased in all groups compared to the inactive group. However, it was significant in those groups that had PD-1 blocker in their formulation, (n=9, P<0.02). **C.** The LDH released from KG-1 cells co-cultured with NK cells only increased in IL-15+Hsp70+PD-1 blocker group that was not significant (n=7, P>0.05). **D.** IFN-γ level in the group that received IL-15+PD-1 blocker significantly increased compared to other groups (P<0.03). Although, it was not significant in comparison to IL-15 treated group (n=7, P>0.05). **E**. *GZMA* and **F.** *GZMB* expression level was increased in IL-15+Hsp70+PD-1 blocker group compared to other groups (n=7, P<0.01). **G.** *PRF-1* was higher in IL-15+Hsp70+PD-1 blocker treated group (n=7, P<0.02). Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical analysis was performed using unpaired two-way Anova. **H.** Fas ligand expression upregulated in activated groups. However, it was over-activated when the NK cells were treated with IL-15+Hsp70 (n=7, P<0.0001). **I.** The expression of the TRAIL gene was upregulated in IL-15+Hsp70 treated NK cells which is just significant compared to IL-15+Hsp70+PD-1 blocker (n=7, P<0.008). **J.** AKT-1 expression level showed a significant increment in the IL-15+PD-1 blocker-treated NK cells compared to other groups (n=7, P<0.01). **K.** The expression level of PIK3CB enhanced in the IL-15+PD-1 blocker-treated NK cells but as shown in the plot there is no significance with IL-15+Hsp70+PD-1 blocker group (n=7, P<0.01). Statistical analysis was performed using unpaired two-way Anova. *; P<0.05, **; P<0.01, ****; P<0.0001, AML; Acute myeloid leukemia, NK; Natural killer cell, IL; Interleukin, Hsp70; Heat shock protein 70, PD-1; Programmed cell death protein 1, LDH; Lactate dehydrogenase, and IFN-γ; Interferon gamma.

Discussion

Reflective of the current study revealed several key findings that help us better understand the role of Hsp-70 and PD-1 blocker-based therapy in patients with AML. Immune checkpoints, including PD-1 and CTLA-4, are novel targets for cancer immunotherapy and made a promising tool in the path of solid cancer treatment (21, 22). PD-1/PD-L1 inhibition has been demonstrated in multiple myeloma patients to improve NK cell-mediated tumor lysis (23, 24). Despite the favorable outcomes of blocking such immune checkpoints, the results were not well studied in haematological cancers. meanwhile, many malignant cells over-expressed heat shock proteins, including Hsp70 and Hsp90, which indicate their crucial role in malignant progression (25, 26). Regarding AML, the overexpression of Hsp70 is identified (27, 28), however, its role is controversial (28). Several studies have reported that Hsp70 can induce cytotoxic activity in NK cells against tumors, including melanoma and glioblastoma (29, 30). We depicted that pre-treatment of NK cells with different combinations of Hsp70 and PD-1 inhibitor may stimulate their anti-tumor effects. However, due to our results, Hsp70 in combination with IL-15 didn't have significant effect on NK cells cytotoxicity potential.

Twenty patients were enrolled in the present study based on inclusion criteria, including: the estimated survival of the patients, which was more than three months, and the traditional treatments were ineffective for these patients, or these patients could not tolerate the traditional treatments. However, eleven patients were excluded based on insufficient blood volume, cell count, and blood clotting. Another two patients' samples were lost during q-RT-PCR. Therefore, the nine patients were used in some experiments, and

in qRT-PCR-related experiments, the seven patients' samples were used. In the present study, the patients who were treated with fludarabine and busulfan entered remission. However, their disease relapsed, and they again were candidates for chemotherapy. Samples were derived from these patients before the second chemotherapy. It should be noticed that the synergistic effect of fludarabine with other drug combinations has been widely used as salvage chemotherapy for refractory/relapsed AML and acute lymphoblastic leukaemia (ALL) (31). As mentioned earlier, twenty patients were enrolled in the present study based on inclusion criteria. However, eleven patients were excluded based on insufficient blood volume, cell count, and blood clotting. Another two patients' samples were lost during q-RT-PCR. Therefore, nine patients' samples were used in some experiments, and seven patients' samples were proper for the qRT-PCR experiment.

Our results indicated that the percentage of NK cells in patients with non-M3 relapsed AML who received fludarabine and busulfan was about 12.3 ± 4.18% of total lymphocytes similar to normal people (31). However, 7.05 ± 1.14% of them expressed PD-1 in the NK cell population that higher than PD-1 expression in normal NK cells (2-5%) (14). The main question of the present study was to evaluate the combinatory effect of Hsp70 and PD-1 blocker to activate NK cells derived from patients with non-M3 relapsed AML. We found that the expression of PD-1 significantly reduced when NK cells activated with a different formula of IL-15, Hsp70, and PD-1 blocker. IL-15 primarily acts as a cell-surface molecule on antigen-presenting cells, which provides IL-15 translocate to mononuclear cells such as NK and CD8 memory cells. Many studies showed that IL-15 induced prolonged expansion and activation of NK cells. Based on these characteristics, IL-15, besides the stimulating activity, could increase the proliferation of NK cells (32). Therefore, we used IL-15 as the basic compound in our study to find the best formulation of NK cell activation. However, the active and expanded clones in groups that received IL-15+PD-1 blocker seemed to be more than other groups. NKG2A, in cooperation with PD-1, exerts its inhibitory activity on NK cells through binding to classical and non-classical MHC class I molecules (32). Our results exhibited that the expression of surface NKG2A in the NK cells treated in IL-15 and IL-15+PD-1 blocker groups, considerably decreased and its expression was increased with adding the Hsp70 to activation media. It is worth mentioning that IL-15 is recommended to be involved in PD-1 blocking and NK cell activation through activation of the PI3K/AKT/mTOR signalling pathway (33, 34), as also observed in our data. The over-expression of NKG2A also was reported by the Fehniger and Caligiuri (35) study, which showed induction of cytotoxicity in NK cells in parallel to increasing NKG2A when the cells were treated with IL-2/TKD (TKDNNLLGRFELSG; a 14-mer Hsp70 subunit). Here our results showed a significant increase in the expression level of *PIK3CB* and *AKT-1*, the downstream molecules of NKG2D and critical regulator of NK cell activation, in the presence of IL-15+PD-1 blocker. However, we did not detect any changes in the level of NKG2D positive NK cells, and a significant reduction was observed in NKP30 and even NKP46 post-treatment of cells by Hsp70 or PD-1 blocker and both of them. Meanwhile, the cytotoxic potential of NK cells was enhanced in groups that received PD-1 blocker, which was concomitant with increasing in releasing of IFN-γ and upregulation of *granzyme A/B* and *PRF1* (16, 36).

Although, several studies reported that a combination of Hsp70 and PD-1 blocker might over-activate NK cells in Fighting cancer cells (37, 38). But our results showed that the presence of Hsp70 as an activating factor in the combination of IL-15 increases the ambiguity of data. For example, Hsp70 in activating media caused an increase in the NKG2A positive cells, reduced IFN-γ releasing, and reduced the expression of *FAS-L* and *TRIAL*. All uncertainties may back to AML-NK cells as a source of NK cells and even the undefined role of Hsp70 in these patient's specific cells (39, 40).

Conclusion

Therefore, based on the results of this study, we suggested that the combination of IL-15 and PD-1 blocker can reactive AML-NK cells, increasing their killing ability against tumor cells, enhancing the key factors in NK cell function and increase releasing of IFN-γ, granzymes as well as perforin. Moreover, we suggested that Hsp70 could act as a disruptive factor to induce cytotoxic NK cells when combined with IL-15 and PD-1 blocker.

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Authors' Contributions

J.F.; Contributed to the conceptualization of the study, data collection, and was a major contributor

to writing the manuscript. A.H., A.A.H.A.; Medical consultant and helped in collecting samples. M.A., N.P., N.S.A., F.Gh.; Contributed to data collection and data analysis. M.S., M.E.; Contributed to the conceptualization of the study, revised the manuscript, and supervised the study. All authors read and approved the final manuscript.

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