Radio-immunotherapy by $^{188}\text{Re}$-antiCD20 and stable silencing of IGF-IR in Raji cells, new insight in treatment of lymphoma

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Research Article

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Abstract

Hematologic malignancies such as Non-Hodgkin's lymphoma (NHL), remain a serious threat to human health due to their heterogeneity and complexity. The inherent genetic heterogeneity of NHL B-cells, as well as the instability of lymphoma cancer cells, results in drug resistance in lymphoma, posing a fundamental challenge to NHL treatment. Burkitt lymphoma (including Raji cell line) is a rare and highly aggressive form of B-cell NHL. Since overexpression of the insulin-like growth factor-1 receptor (IGF-1R) playing a prominent role in the development and transformation of different malignancies, especially lymphoma malignancies, we have explored the role of IGF-1R in the development and progression of Raji cells and the stable silencing of IGF-1R by lentivirus-mediated RNA interference (RNAi). We have shown that stable silencing of the IGF-1R gene in Raji cells using lentivirus-mediated RNAi have resulted in a significant reduction in Raji cell proliferation. Moreover, the results of the cell viability assays indicated high resistance of Raji cells to rituximab. However, coupling rituximab to 188Re potentially leads to specific targeting of Raji cells by 188Re, improving the therapeutic efficacy. We found that the synergistic effect of using a gene therapy-based system in combination with radioimmunotherapy could be a promising therapeutic strategy in the future. To the best of our knowledge, this is the first study that reports the knock down of IGF-1R via lentiviral-mediated shRNA in Raji cells.

Introduction

Hematologic malignancies, blood cancers, remain a serious burning issue and threat to human health due to their heterogeneity and complexity. Lymphoma is a type of blood malignancy affecting lymphocytes and includes Hodgkin lymphoma and non-Hodgkin lymphoma (NHL). NHL is a heterogeneous group of lymphoproliferative malignancies that is more common in developed countries and is the most common blood cancer worldwide [1, 2]. Burkitt lymphoma is a relatively rare and highly aggressive form of B-cell NHL. Raji cells are a human B lymphoblastoid cell line derived from a Burkitt lymphoma patient. Chemotherapy, radiation therapy, as well as a combination of these therapies, are common NHL methods of treatment. However, the emergence of primary or secondary chemoresistance as well as immunoresistance has seriously challenged the successful treatment of disseminated B-cell lymphomas in the clinic and is a leading cause of mortality in NHL patients [3–5]. Targeted anti-cancer therapies based on monoclonal antibodies (mAbs) against antigens on the surface of tumor cells, has received special attention. Rituximab is the first anti-CD20 chimeric monoclonal antibody to be approved by FDA for the treatment of NHL. The anti-cancer effects of rituximab are exerted by targeting CD20 antigen on CD20+ B-cells primarily by induction of apoptosis, complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. However, rituximab has limited therapeutic effect as well as relapse and adverse side effects due to its resistance to treatment. Only 40% of patients who initially respond to rituximab are capable of responding during relapse, as according findings [6–9]. Therefore, successful treatment for the NHL requires rational design of new methods that overcome drug resistance while still demonstrating considerable therapeutic efficacy. Generally, various factors such as insulin-like growth factors (IGFs) as potent mitogens are involved in the invasion and metastasis of many tumor
cells, and overexpression of these factors and their receptors leads to malignancies like NHL. Insulin-Like Growth Factor 1 Receptor (IGF-1R) is a transmembrane tyrosine kinase glycoprotein expressed on the surface of human cells that is activated by insulin-like growth factor 1 (IGF-1). IGF-1R plays a fundamental role in tumor transformation as well as tumor cells survival [10–13]. As a corollary, the IGF-1R gene has emerged as an appealing and suitable target for the development of new anti-cancer treatments in order to targeted therapy of human malignancies [14–17]. Recently, targeted molecular therapies have been developed and employed as an effective and targeted therapeutic strategy to suppress tumor cells. The capability of RNA interference (RNAi) to target and block the expression of factors involved in human malignancies has received a lot of attention in recent years [18]. We previously demonstrated that Lenti-shRNA-mediated stable downregulation of IGF-1R can significantly inhibit the growth and proliferation of human embryonic kidney cells (HEK293T) [19]. In the present study, our main purpose was to investigate the reduction of tumor growth and potency while also increasing apoptosis induction in lymphoma cancer cells (Raji) utilizing an RNAi expression system based on the Lenti-shRNA structure to target and knock down IGF-1R in combination with Rhenium 188-anti CD20 radioimmunotherapy. Rhenium-188 as a β-emitting radionuclide due to its unique nuclear properties has attracted special attention for diagnostic and therapeutic applications in cancer therapy. Rhenium 188 has a tissue penetration of 10 mm as well as a short half-life (16.9 h), making it more suitable for in vivo applications [20, 21]. Furthermore, different downstream targets may elicit various biological responses, that could be employed as biomarkers to predict IGF-1R therapeutic outcomes [22]. Therefore, downstream elements of the IGF signaling axis like BCL-2, including PI3K/AKT/mTOR pathway are important in determining cell apoptosis and survival [23–25]. Here, we also evaluated the BCL-2 expression as a downstream biomarker involved in the IGF signaling and rituximab mechanism of action and determine its correlation with the IGF-1R knockdown and rituximab treatment.

**Material And Methods**

HEK293T cells (human embryonic kidney, ATCC CRL-3216), Raji cells (ATCC® CCL-86™), and Daudi cells (ATCC® CCL-213™) (prepared from Pasteur Institute of Iran), Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, USA), Roswell Park Memorial Institute medium (RPMI-1640; Gibco, USA), Fetal Bovine Serum (FBS; Gibco, USA), streptomycin (Invitrogen, Carlsbad, USA), pGIPZ lentiviral vector (GE Healthcare Dharmacon company), psPAX2 plasmid (Addgene, Cat #12260), pMD2G plasmid (Addgene, Cat #12259)), GeneJET Plasmid Midiprep Kit (Thermo Fisher Scientific, USA), RNAsay ® Plus Mini Kit (QIAGEN Kit, Germany), Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA), RIPA lysis buffer (Cell Signaling Technology, Beverly, MA, USA).

**Cell culture:**

The HEK293T cells were cultured in DMEM high glucose medium supplemented with 10% FBS, and 1% penicillin-streptomycin and incubated at 37 °C with 5% CO2 and 95% air condition. Moreover, Raji and Daudi human lymphoma cells were cultured in RPMI medium supplemented with 10% FBS, and 1%
penicillin-streptomycin and incubated in a same condition. As well as, the trypan blue dye exclusion assay was used to evaluate the viability of the cells.

**Evaluation of IGF-1R and CD20 genes expression in Raji and Daudi lymphoma cell line**

Raji and Daudi lymphoma cell lines were being investigated for IGF-1R gene expression using the RT-PCR technique. Cells were cultured with at density of $10^5 \times 4$ in RPMI medium supplemented with 10% FBS, and 1% penicillin-streptomycin in cell culture flask. After reaching 80% confluency, cells were collected and RNA was extracted using the RNeasy Plus Mini Kit (Q74134) according to the guidelines of the kit. The RNA concentration was measured by Nano drop and verified by agarose gel electrophoresis. The cDNA synthesis was performed using the ReverseAid First Strand cDNA Synthesis Kit. Finally, the expression of the IGF-1R and CD20 genes in Raji and Daudi lymphoma cell lines were evaluated and compared using the RT-PCR technique.

**Lentiviral vector preparation**

pGIPZ lentiviral vector containing shRNA against the human IGF1R gene (GenBank accession No. 000875.4) carrying the 5'-TAGAAATGACAGTTCTCTC-3' sequence targeting the 3399-3417 region on the IGF1R gene (Figure1) and pGIPZ non-silencing shRNAmir lentiviral vector (as a negative control) were purchased from the GE Healthcare Dharmacon Inc. The negative control was a scrambled sequence, 22mer: 5'-ATCTCGCTTGGGCGAGAGTAAG- 3', that had no notable homology to human gene sequences.

**Production of recombinant lentivirus**

The cassette containing GIPZ-shRNA IGF1R-turbo green fluorescent protein (tGFP) as well as another cassette carrying scrambled negative control were cotransfected with psPAX2 and pMD2G into HEK293T cells by reverse transfection method using Lipofectamine 3000 reagent. Each transfection sample were prepared separately in two solution A and solution B. First, 500 μL of Opti-MEM I Reduced Serum Medium was added to both solutions A and B. The vectors GIPZ, pMD2 and psPAX2 were added to solution B with concentrations of 1.7, 3.3 and 1.7 μg and 13 μL of P3000 Enhancer reagent, respectively. Then, 15 μL of Lipofectamine 3000 reagent was added both solutions A and B. Following, HEK293T cells were added a with a density of 2.5×106 to the wells in a 10cm² plate containing mixture of solutions A and B and incubated. The transfection medium was replaced with fresh medium after 14 h of transfection. Based on the expression of tGFP 24 hours, 48 hours, and 72 hours after transfection, fluorescence microscopy was used to confirm the packaging of the recombinant lentiviruses. Flow cytometry analysis was used to determine the lentivirus titer based on tGFP expression. Finally, the packaged recombinant lentiviruses were harvested in triplicate from the supernatant of the cells (centrifuged for 5 min at 2000 rpm and 4°C) subsequently filtering through a 0.22 μm filter unit and stored at -80°C.

**Virus titration**
HEK293T cells were seeded with density of 5×10^5 cells per well in a 12-well plate containing 1 ml of DMEM-10 medium, and the media was discarded the next day. The virus supernatant was prepared in five serial dilutions (10^-4-1 μl), and the volumes were increased to 500μL via adding DMEM-10 to the wells in duplicate. The seeded plate was incubated at 37°C in 5% CO₂. The cells were harvested and analyzed for GFP expression employing flow cytometry, on the fifth day. The dilution that yielded in 1-20% GFP positive [26] result was selected, and the virus titer was calculated using the following standard equation:

“Titer (HEK293T transducing units/ml) = (Number of target cells on day 1) × (% of GFP-positive cells/100) /volume of the supernatant (ml)” [26].

In both groups, the recombinant virus titer was 1.5×10^7 IU/mL, demonstrating that the transfection was successful.

**Raji cells transduction**

Since Raji cells are non-adhesive and hard to transduce cells, the spinfection method was used to transduction of Raji cells. First, cell suspension was collected, counted and prepared for spinfection. Subsequently, 100 µL of medium containing 2 × 10^5 cells with 100 µL of recombinant lentiviruses at a high multiplicity of infection (MOI = 10) were transferred to a microcentrifuge tube and centrifuged at 1600 rpm and 32 °C for 1 h. The cell precipitate was then thoroughly resuspended with the supernatant, and each tube was transferred to a well in a 24-well plate and final volume was reached to 1 ml using complete RPMI medium. The tGFP expression was analyzed in Raji cells to investigate the transduction efficiency of recombinant lentiviral vectors. The fluorescence of tGFP was examined under a fluorescent microscopy (OPTIKA Model IM-3FL4, ITALY), and the obtained images were analyzed using Image-J software.

**Determination of optimal puromycin concentration**

Since each cell line responds to puromycin selection differently, therefore the optimal puromycin concentration for the Raji cell line is required to be determined. Determination of puromycin concentration was performed to obtain the minimum concentration in which all cells killed. To determine the optimal concentration, different concentrations of puromycin (0.5 - 10 µg/mL) were evaluated and finally the optimal concentration of puromycin was determined about 2 µg/mL for Raji cells.

**Quantitative reverse transcription Polymerase Chain Reaction (RT-qPCR)**

Following transfection, the knockdown effect was evaluated employing reverse transcription and real-time PCR. The total RNA from each cell group was extracted using the RNeasy®Plus Mini Kit (QIAGEN Kit, Germany), three to seven days after infection with lentiviruses. The reverse transcription (RT) reaction was performed employing Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). The primer sequences were as follows: IGF1R Forward: 5’-TCTGTGTTTCTTATGcca- 3’ Reverse: 5’-CCTGCTGGTATTCTTCTTCTAGG- 3’ (product length=145 bp) BCL2 Forward: 5’-
ACTGGAGAGTGCTGAAGATTG- 3’ Reverse: 5’ - CAGCATGATCCTCTGTCAAGT- 3’ (product length=88 bp), and GAPDH was used as an internal control: Forward: 5’ - ATTATTCTCTGATTTGGTCTAT- 3’ Reverse: 5’ - TCCTGGAAAGATGGTGATG - 3’ (product length=218 bp). The cutoff (Ct) for each of the samples was drawn on a standard curve, and the copy numbers of mRNA were calculated. The relative IGF-1R mRNA levels were expressed as a proportion of IGF-1R and BCL-2 to GAPDH by the REST software. The results were reported in triplicate, and the data were indicated as mean ± SD.

**Western blot analysis:**

Cells were lysed by RIPA lysis buffer according to the guidelines of the manufacturer. Briefly, the RIPA lysis buffer was added to the cell pellets, followed by 30 min incubation on ice with repeated vortexing and final centrifugation at 10,000 g for 10 min. The Bradford assay was used to determine the protein concentrations in the samples. The IGF1-R, BCL-2, and β-actin protein lysates (30µg) were resolved on 5-10% SDS-PAGE gels and transferred to polyvinylidenfluorid (PVDF) membranes. The membranes were blocked for 60 min at room temperature using a blocking buffer made up of 5% non-fat dry milk dissolved in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T). The membranes were immunoblotted overnight at 4°C with 1:1000 (0.5 µg/ml) primary rabbit monoclonal anti-human antibodies to IGF1-R, BCL-2, and β-actin following washing with TBS-T. The membranes were washed three times using TBS-T and then incubated for an hour at ambient temperature with goat anti-rabbit secondary antibody conjugated to HRP (horseradish peroxidase) at 1:3000 dilution. The ECL Western blotting detection system (Pierce, USA) was used to identify protein-antibody complexes. The Image J analysis software was employed to perform the densitometry analysis. In this experiment, β-actin protein was used as the control gene. Each experiment was performed in triplicate to ensure that the results remained reliable.

**Radiolabeling of anti-CD20 with Re-188**

Rituximab, the first FDA-approved anti-CD20 monoclonal antibody, was used in this study. The Rituximab mAb (10 mg) was reduced by reaction with 10 µL of 2-mercaptoethanol at room temperature for 30 min. Then, the obtained solution was purified by PD-10 purification column using buffered saline phosphate (PBS) (pH 7.4). The optical density at 280 nm on a UV/visible spectrophotometer was used to measure the concentration of the reduced antibody. To Labeling of anti-CD20 with $^{188}$Re the following reagents were used; 1 mg of anti-CD20, 82.8 mg of sodium tartrate, 1 mg of SnCl$_2$ and 0.25 mg of gentisic acid. Finally, Perrhenate (Na$^{188}$ReO$_4$) eluted from the $^{188}$W/$^{188}$Re generator (Polatom) in 0.9% saline (0.9% NaCl) was added and then resulting solution was incubated for 1 hour at room temperature. Evaluation of labeling efficiency was performed using instant thin-layer chromatography (ITLC). Solvents used include normal saline, acetone, and water/ methanol/ ethanol composition. To evaluate the stability of the rituximab-Re$^{188}$ compound, 1 ml samples of fresh human blood serum were prepared, and then 100 µCi/ 100 µl rituximab-Re$^{188}$ was added to each and incubated at 37 ° C. Stability of samples at 2, 4, 24 and 48 hours after incubation was determined by TLC.
**Cell survival assays**

**MTT assay**

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate the proliferation of Raji cells against different groups. The proliferation of Raji cells was measured in the following treatment groups: rituximab at 0, 10, 20, 80, 100, 200 µg/ml for 24, 48 and 72 h incubation; rituximab-Re$_{188}$ at 0, 1, 5, 10, 20 and 40 µg/µCi for 24 and 48 h incubation; Re$_{188}$ at 0, 1, 5, 10, 20 and 40 µCi for 24 and 48 h incubation. The proliferation of Raji cells with knock down of IGF1-R were also evaluated following treatment with rituximab-Re$_{188}$ at 0, 1, 5, 10, 20 and 40 µg/µCi for 24 and 48 h or Re$_{188}$ at 0, 1, 5, 10, 20 and 40 µCi for 24 and 48 h. The proper controls including Raji cells transduced with shRNA against IGF1-R, Raji cells transduced with shRNA non silencing (control) and Raji cells (without treatment) were also evaluated for 24, 48 and 72 h incubation. Cells were seeded in a 96-well plate at a standard density (1 x 10$^4$ cells per well) in RPMI medium supplemented with 10% FBS. Since Raji cells are non-adherent and suspension cells, no overnight incubation was performed, and the selected different groups were treated immediately upon seeding. They were then incubated at predetermined time periods for different concentrations. After treatment period, 10 µl of MTT solution (5 mg/ml) was added and the plates were incubated for 3-4 hours. The plates were then incubated for 30 min with 100 uL of DMSO solution followed by 5 min shaking to thoroughly dissolve the MTT formazan in the DMSO. The absorbance of the wells was recorded using an Eliza reader at 570 nm wavelength.

**XTT assay**

The XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay was used to evaluate the effects of rituximab on the survival of Raji cells using Cell Proliferation kit II (Roche company), since it is more sensitive than the MTT test and is more validated for non-adherent cells like Raji cells. XTT is a tetrazolium compound that is reduced by mitochondrial enzymes found only in metabolically active live cells and turned into a water-soluble orange compound. The number of live cells is determined by the amount of orange compound produced.

**CD20 expression analysis by flow cytometry**

The level of CD20 expression in Raji cells was evaluated to determine the mechanisms of rituximab resistance in Raji cells. Flow cytometry analysis was used to evaluate the level of CD20 expression in Raji cells treated with different concentrations of rituximab, including 10, 20, 40, and 80 µg/ml.

**CD20 sequencing and bioinformatics analysis**

CD20 gene sequencing was performed to investigate for mutations occurrence or possible changes in the transcript of this gene. RNA was extracted from Raji cells, cDNA was synthesized, as well as the expression of the CD20 gene was evaluated using RT-PCR. RT-PCR products were electrophoresed on 2% agarose gel and used for sequencing. The DNA and amino acid sequences of the CD20 gene were
studied using a variety of methods and software once it was sequenced. The "expasy" and "Clustal Omega", available at https://web.expasy.org/translate and https://www.ebi.ac.uk/Tools/msa/clustalo, were used to analyze and compare DNA and amino acid sequence alignment, respectively.

**Statistical analysis:**

All assays were performed in three replicates, and the IGF1R to β-actin ratios demonstrated as mean±SD. Graphpad Prism 9 software was used to compare intergroup statistical differences using the independent sample t test and one-way analysis of variance (ANOVA). The significance level was considered at p<0.05.

**Results**

**Evaluation of IGF-1R and CD20 genes expression in Raji and Daudi lymphoma cell lines**

The Raji and Daudi cell lines were evaluated for the expression of IGF-1R gene at the mRNA level using the RT-PCR technique in order to determine the cell line with the highest expression level. The electrophoresis results of RNAs extracted from the Raji and Daudi cells showed good RNA extraction quality. Subsequently, using the same concentrations of extracted RNAs, cDNA was synthesized and the expression of the IGF-1R gene was evaluated using the RT-PCR technique. As well as, GAPDH gene was employed as Housekeeping gene or control sample in this assay. The results of the RT-PCR test were analyzed using ImageJ software in order to select the cell line with high expression of the IGF-1R gene. The electrophoresis results illustrate that the IGF-1R gene band in the Raji cell line has a higher resolution than the Daudi cell line, indicating high expression of IGF-1R gene in Raji than Daudi. Finally, the Raji cell line was selected as the target cell exhibiting high expression of the IGF-1R gene based on the results of the RT-PCT test. Furthermore, to validate the expression of the CD20 gene in Raji cells, the RT-PCR test was performed, which revealed that this gene was highly expressed in Raji cells.

**Production of recombinant lentivirus**

Based on the expression of tGFP at 24, 48, and 72 hours after transfection, fluorescence microscopy was employed to confirm the packaging of the recombinant lentiviruses. More than 90% of HEK293T cells were transduced by pGIPZ lentiviruses, as illustrated in "Figure 2." The titer of lentiviruses was determined to be approximately $1.5 \times 10^6$ IU/mL in all groups based on the expression of tGFP.

**Raji cells transduction**

To evaluate the transduction efficiency of recombinant lentiviral vectors in Raji cells, tGFP marker expression was examined using fluorescence microscopy and flow cytometric analysis. The frequency of positive Raji cells-tGFP was analyzed 72 h after transduction. It was found that the percentage of Raji cells expressing tGFP increased significantly after 72 h of transduction (over 90%) (Figures 3 and 4).

**Evaluation of IGF-1R and Bcl-2 genes expression in Raji cells**
The real-time PCR technique was employed to evaluate the expression of IGF-1R and Bcl-2 genes in Raji transduced cells. Analysis of results by the REST software indicated that the IGF-1R expression was significantly downregulated in cells with recombinant lentivirus-mediated shRNA expression vectors (83%) but not in non-silencing (Raji-NS) and normal groups (Figure 5). According to the REST software, downregulation of IGF-1R expression drastically decreased BCL2 gene expression (63%) at the mRNA level in Raji shIGF1R cells as compared to Raji negative control cells (Figure 5). Comparing Raji-shIGF1R and Raji-NS cells, the ANOVA analysis revealed a statistically significant reduction in expression.

**Western Blot**

The western blot assay was employed to determine the levels of IGF-1R and BCL-2 proteins in transduced Raji cells, revealing IGF-1R and BCL-2 silencing at the post-translational level. Regarding Raji cells transduced with the pGIPZ-IGF1R recombinant vector, the densitometric analysis revealed significantly reduced levels of IGF-1R (67%) and BCL2 (52%) proteins compared to Raji cells (non-transduced cells) and non-silencing (Ns) negative controls. These findings showed that pGIPZ-IGF1R efficiently suppressed IGF-1R at the RNA and protein levels, as well as affecting other genes involved in the apoptotic pathway, such as BCL-2 (Figure 5).

**Radiolabeling of anti-CD20 with Re-188**

Following labeling of rituximab (anti-CD20) with $^{188}$Re, thin-layer chromatography (TLC) was used to evaluate the radiochemical purity of $^{188}$Re-anti-CD20. Radiochemical purity evaluation showed excellent labeling yield (over 96%) of the rituximab (Figure 6 A, B). Moreover, the stability of $^{188}$Re-anti-CD20 was determined in cold temperature (4 ºC), room temperature (RT), phosphate buffer and human blood serum at time periods from 2 h to 48 h. The stability of $^{188}$Re-anti-CD20 is maximal during the 2-h incubation time, as illustrated in Figure 6 C. Additionally, as compared to other conditions, $^{188}$Re-anti-CD20 has excellent stability at cold temperature (4 ºC).

**Cell survival assays**

**MTT assay**

The effect of different concentrations of rituximab (0, 10, 20, 80, 100 and 200 µg/ml) were evaluated on Raji cells survival and proliferation during three time points of 24, 48, and 72h. The results revealed that the selective concentrations of rituximab had no deleterious effect on Raji cell viability (Figure 7A). According to previous studies, more than 50% of lymphoma patients are resistant to rituximab due to a variety of faults with drug mechanisms of action [27, 28]. Then we evaluated the effects of rituximab- Re$^{188}$ and Re$^{188}$ on the survival of non-transduced Raji cells at 24 and 48 h. Since the half-life of rhenium radioisotope is around 17 h, evaluating the effect of rhenium in the first 24 h is more reliable. Both Re$^{188}$ and rituximab- Re$^{188}$ compounds suppressed non-transduced Raji cell growth, as shown in Figure 8 A, B although the reduction effect of rituximab- Re$^{188}$ on cell survival was higher than rhenium 188 alone. The
effect of different concentrations of rituximab- Re\textsuperscript{188} and Re\textsuperscript{188} was also evaluated on survival and growth of transduced Raji cells with shRNA against IGF1-R at 24 and 48 h (Figure 8 A, B). Both rituximab-Re\textsuperscript{188} and Re\textsuperscript{188} reduced the growth of Raji cells in 24-h treatment; however, the negative effect of rituximab- Re\textsuperscript{188} on cell survival was greater than that of Re\textsuperscript{188}. Furthermore, the effect of rituximab-Re\textsuperscript{188} and Re\textsuperscript{188} on the survival of transduced Raji cells was greater than that of non-transduced cells (Figure 8 A, B. The MTT assay results showed that rituximab- Re\textsuperscript{188} had a greater impact than Re\textsuperscript{188} in suppressing Raji cell growth and survival during 48 h of treatment (Figure 8 B).

**XTT assay**

XTT assay was performed to evaluate the sensitivity of Raji cells to rituximab (5, 10, 50 and 100 μg/ml). As shown in Figure 7A, the results of the XTT assay similar to MTT assay revealed that Raji cells were highly resistant to different concentrations of rituximab (Figure 7B).

**CD20 expression analysis by flow cytometry**

The levels of CD20 expression in Raji cells was determined by flow cytometry analysis. Despite the expectation of 90% CD20 expression in B-NHL cells, the flow cytometry results showed an expression of 44.2% of the CD20 marker in Raji cells, indicating a reduction in expression. Moreover, alterations in the level of CD20 expression exposed to different concentrations of rituximab including 10, 20, 40 and 80 μg/ml, demonstrating that increasing the rituximab concentration led to the reduction of CD20 expression levels (Figure 92).

**Bioinformatics analysis**

Since the CD20 is the target antigen of the rituximab antibody in Raji cells, mutations in the CD20 gene or alterations in protein folding are among the major causes for the lack of correct binding of the rituximab antibody to the CD20 antigen. In this regard, to investigate the possibility of mutation occurrence in the CD20 gene in Raji cells, the expression of this gene was assessed and confirmed using RT-PCR assay and then the CD20 gene sequencing was performed. The RT-PCR product of CD20 gene was sequenced according to the exact location of the rituximab epitope on the CD20 protein. No mutations were found in whatsoever area of the CD20 sequence, especially in the epitope regions, following analysis and comparison of sequencing results (Figure 10).

**Discussion**

Resistance to conventional therapies such as chemotherapy and radiation therapy in the treatment of NHL cancer has posed a significant challenge to effective NHL treatment. This has led to the development of new gene therapy-based treatment strategies that downregulate oncogenes and also increase the sensitivity of cancer cells to radiation and chemotherapy [3, 4, 29]. As according different studies, IGF-1R activity signaling pathways potentially induce chemoresistance in hematological and other malignancies [13, 15, 30]. In this regard, we previously employed six lentivirus-mediated shRNA-
targeting IGF-1R human gene structures to knockdown IGF1R in HEK 293T cells in six different positions in order to find an effective cassette for the IGF-1R knockdown (V2LHS-131071) [19]. Lentivirus constructs were miR-30 adaptive shRNAs increasing the efficiency of the gene-silencing transcript. This strategy, on the other side, has also been shown to generate matured siRNAs that are significantly efficient [31]. Here we reported the efficiently silencing of the IGF-1R gene using RNAi expression system based on the Lenti-shRNA both at the mRNA and protein levels in Raji cells. In this work, the expression level of the IGF1R gene at the mRNA level was significantly reduced (83%) compared to Raji-shNS cells, similarly to our previous study employing the Lenti-shRNA structure. Moreover, as compared to Raji-shNS cells, the expression level of IGF-1R protein in Raji-shIGF1R cells reduced up 67%. As well as, the findings indicated a significant decrease in Bcl2 gene expression up to 63% at the mRNA level and 52% at the protein level in Raji cells. The findings imply that knock-down endogenous expression of IGF-1R is efficiently performed using lentivirus-mediated RNAi system in comparison with antisense RNA, dominant negative and immunotherapy strategies. As also illustrated by Wang et al. the applying of lentivirus-mediated shRNA results in roughly 56.8 and 61.2% reduction in the expression levels of IGF-1R mRNA in Saos-2-shIGF-1R and MG-63-shIGF-1R cells. Additionally, in comparison to control cells, the expression of the IGF1R protein in Saos-2-shIGF-1R or MG-63-shIGF-1R cells were also decreased by 47.7 and 54.6% [23]. In another attempt, the expression of IGF-1R mRNA and protein levels in the lung squamous carcinoma cells was reduced by 55% and 69% employing Lenti-shIGF-1R, respectively [32]. The effect of lentivirus-mediated IGF-1R RNAi on Raji cells proliferation was evaluated using the MTT assay, which indicated that treatment of Raji cells with IGF-1R RNAi inhibited cell proliferation in a time-dependent manner (36.3% at 24 h and 42.9% at 48 h). Some other study revealed that the LV-IGF1R shRNA treatment with breast cancer cells decreased cell proliferation by 37.3% and 43.5% compared to the control group at 48 and 72 h, respectively [33]. In addition, the effect of IGF-1R inhibition on BCL2 expression was examined, and the results revealed that Bcl-2 mRNA and protein levels were significantly reduced. The BCL2 mRNA and protein expression in Raji-shIGF-1R cells was decreased by nearly 63% and 52% compared to Raji-shNs cells, respectively. Increased expression of BCL2 anti-apoptotic protein is involved in the pathogenesis of many non-Hodgkin's lymphomas. It has been reported that the increased expression of BCL2 in 85% of follicular lymphomas and 30% of diffuse B cell lymphomas and in 50% of cancers. The findings suggested that the strategy of IGF1R inhibition decreasing proliferation of Raji cells is associated with its contribution to the induction of apoptosis, although the underlying mechanisms need to be investigated further in additional research. Similarly in a study by Wang et al., the anti-apoptotic BCL2 protein was downregulated by 39.2 or 44.6% in two types of osteosarcoma shIGF1R cells compared to the control cells [23]. Another interesting finding in our study is complete resistance of Raji cells to rituximab which proved by MTT and XTT assays. Unexpectedly, different doses of rituximab, ranging from 2.5 µg/ml to 200 µg/ml, were found to have no effect on Raji cells proliferation. While $^{188}$Re alone and $^{188}$Re-rituximab resulted in significant reductions in the proliferation and growth of Raji cells. $^{188}$Re has attracted a lot of attention and is becoming a potentially attractive alternative in therapeutic nuclear medicine in cancer treatment, due to its excellent characteristics [$t_{1/2} = 16.9$ h, $E_{\text{β}_{\text{max}}} = 2.12$ MeV, $E_{\text{γ}} = 155$ keV (15%) [20]. So far, different antibodies were labeled with $^{188}$Re and evaluated pre-clinically
in a wide range of tumors. For example, in an attempt by Decker et al. evaluated the $^{188}$Re-labeled anti-CD52 monoclonal antibody alemtuzumab for radioimmunotherapy of B-cell chronic lymphocytic leukemia [34]. Generally, despite that the efficacy of rituximab in the treatment of B-cell-associated malignancies has been well established, some patients show resistance to first-line treatment with rituximab, while others experience relapse following the initial response to treatment [35, 36]. Although resistance to rituximab has been investigated in many studies, the exact mechanisms for developing resistance to treatment are still unclear. Different possible mechanisms of resistance have been hypothesized so far, including inhibition of the pathways involved in rituximab’s mechanism of action, i.e. CDC, ADCC, and apoptosis [35, 37, 38]. Since CD20 is the target antigen of rituximab, loss of CD20 antigen, as a result of exposure to the rituximab is another mechanism for rituximab resistance, as seen in lymphoma cell lines and in biopsy samples from relapsed patients [39, 40]. However, decreased expression of the CD20 gene alone could not lead to resistance to rituximab. As illustrated by Tsai et al. targeting CD20 by siRNA only results in a 15% reduction in rituximab-complement-mediated cytotoxicity (CMC). Moreover, no mutations were found in the CD20 gene sequence despite resistance to rituximab [41]. In this regard, CD20 expression analysis in Raji cells by flow cytometry analysis showed 44.2% CD20 expression, which indicates a decrease in CD20 expression. It was found that increasing the concentration of rituximab leads to a further decrease in CD20 expression. Additionally, we evaluated the expression of CD20 gene in Raji cells using RT-PCR technique, which confirmed the expression of CD20 gene in Raji cells., the RT-PCR product of the CD20 gene was sequenced to investigate the occurrence of mutations in CD20, and analysis of CD20 DNA and amino acid sequences demonstrated no mutations. Epigenetics alterations may lead to acquired resistance to rituximab as well [42]. Therefore, it seems that more investigations are required to determine the exact mechanism of resistance of Raji cells to rituximab.

Conclusion

The findings of this study demonstrated that the reduction of IGF1R expression is the key to the sensitivity of lymphoma cells to radiotherapy and radioimmunotherapy. Since no clinical trial has been performed to evaluate the efficacy of combined radioimmunotherapy treatment (monoclonal anti-CD20 antibodies coupled with radionuclide) with IGF1R inhibitors, it is critically necessary to perform. The major argument is that future cancer treatments will probably require a combination of strategies in overcoming rituximab resistance.

Declarations

Author contributions

All authors contributed to the study conception and design. Conceptualization, data curation, formal analysis were done by Mohammad hossein Ghahremani and Leila Nasehi. Methodology was done by Leila Nasehi, Baharak Abdolhosseinzadeh and Hossein Rahimi. original draft was done by Leila Nasehi
and Mohammad hossein Ghahremani. Writing—review & editing was done by Leila Nasehi and Mohammad Hossein Ghahremani. All authors read and approved the final manuscript.

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**Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this article.

**Consent for publication**

All authors gave their consent for this paper publication.

**Ethics approval and consent to participate**

The research was approved by the ethics committee of Biomedical Research of Tehran University of Medical Sciences (Ethics Code: IR.TUMS.REC. 1394 .473).

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Figures

Figure 1

pGIPZ lentiviral IGF1R shRNA vector
Figure 2

Images (A, B) showing of GFP expression intensity in HEK293T cells after transduction with pGIPZ lentiviral IGF1R shRNA vector.

Figure 3

Transduction of Raji cells with (A) pGIPZ-IGF-1R, and (B) pGIPZ Non-silencing (Scrambled).
Figure 4

Flow Cytometry analysis. (A and B) The graphs demonstration of flow cytometry analysis for pGIPZ Non-silencing and pGIPZ-IGF-1R transduction. Flow cytometry analysis showed transduction efficiency over 90% for pGIPZ Non-silencing and pGIPZ-IGF-1R.
Figure 5

Evaluation of gene expression by Real-time PCR. (A) Quantitative assessment of IGF-1R, and (B) BCL-2 genes expression in transduced Raji cells with shRNA-IGF-1R. Raji; Non transduced cells, Raji-NS; Non silencing transduced cells. Western blot analysis (protein bands). The decreased expression of IGF-1R (C) and Bcl-2 (D) proteins in transgenic Raji cells were confirmed by Western blot results. Raji; Non transduced cells, NS; Non silencing transduced cells.
Figure 6

Displacement of labeled anti-CD20 with $^{188}$Re (A) and displacement of $^{188}$ReO2 (B) in normal saline. In vitro stability of $^{188}$Re-rituximab after incubation in 4°C, room temperature, phosphate buffer and human serum at different time periods (C).
Figure 7

MTT assay results for rituximab in 24, 48 and 72 h on Raji cells. Treatment of Raji cells with different concentrations of rituximab showed no significant reduction in the proliferation of the Raji cells (A). Evaluation of cell viability by XTT assay. Similar to MTT assay the XTT assay results indicated that the Raji cells were highly resistant to rituximab (B).

Figure 8
Investigation of Raji cells viability and proliferation via MTT assay. A) Evaluation of Raji cells proliferation in treatment with Re$^{188}$ and Re$^{188}$-Rituximab in transduced and non-transduced cells in 24 h, and B) the same treatment in 48 h incubation.

**Figure 9**

Determination of CD20 marker levels in Raji cells via flow cytometry analysis. (A, B) The graphs illustration of CD20 expression levels in treatment with different concentrations (10, 20, 40 and 80 μg/ml) of rituximab.
Figure 10

Investigation and alignment proprietary position and location of rituximab epitope on CD20

Supplementary Files

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