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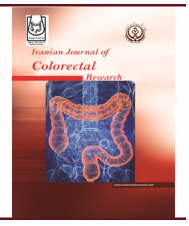

Evaluation of the Synergistic Effects of Photobiomodulation and Natural Killer Cell Therapy in a Colorectal Cancer Xenograft Mouse Model

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Abstract

Background: Colorectal cancer (CRC) is a major cause of cancer-related mortality worldwide, highlighting the need for more effective therapeutic approaches. This study aimed to evaluate the synergistic antitumor effects of combining natural killer (NK) cell-based immunotherapy with photobiomodulation (PBM) in a CRC xenograft model.
Methods: Human peripheral blood-derived NK cells were isolated, and their cytotoxic activity was confirmed in vitro against HT-29 CRC cells. Nude mice bearing HT-29 xenografts were assigned to four groups: control, PBM (808 nm), NK therapy, and combined NK + PBM treatment. Tumor size was monitored throughout the study, and excised tumors were analyzed histologically for NK cell infiltration, mitotic index, and apoptosis.
Results: Both NK therapy and PBM alone significantly reduced tumor growth compared to the control. The combined NK + PBM group demonstrated the greatest suppression of tumor progression. Histopathological analysis revealed markedly enhanced NK cell infiltration, decreased mitotic activity, and increased apoptosis in the combination group compared to the monotherapy groups.
Conclusion: NK therapy and PBM each exert anti-tumor effects in CRC xenografts; however, their combination produces a superior therapeutic response. PBM may serve as a promising adjuvant to enhance NK-based immunotherapy in CRC.

Keywords: Colorectal Neoplasms; Natural Killer Cells; Low-Level Light Therapy; Immunotherapy

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related death worldwide, with an estimated 1.9 million new cases and approximately 935,000 deaths in 2020, according to GLOBOCAN data (1). Despite significant advances in surgery, chemotherapy, and targeted therapies, CRC remains a major clinical challenge due to treatment resistance, recurrence, and metastasis (2).

The immune system plays a central role in tumor surveillance, with natural killer (NK) cells serving as key effectors of innate immunity (3). NK cells can directly eliminate malignant cells through cytolytic granules, such as perforin and granzymes, and via Fas/FasL signaling. They also mediate antibody-dependent cellular cytotoxicity (ADCC) through engagement of the CD16 receptor (4). Importantly, impaired NK cell infiltration or function in CRC is associated with poor prognosis (5). These findings highlight the potential of NK cell-based immunotherapy as a promising approach for CRC treatment.

Photobiomodulation (PBM) is a non-invasive therapy that uses red or near-infrared light to modulate cellular activity through mitochondrial stimulation (6). PBM affects normal and malignant cells differently, partly due to the Warburg effect. In healthy cells, PBM induces a controlled increase in mitochondrial activity, leading to the generation of reactive oxygen species (ROS) that activate transcription factors such as NF-κB and Nrf2, thereby promoting the expression of antioxidant and cytoprotective genes (7). Conversely, in cancer cells, PBM can exacerbate metabolic stress and trigger apoptosis, demonstrating its potential as an anti-tumor therapy.

At the molecular level, PBM influences gene expression through multiple mechanisms. Red and near-infrared light stimulate mitochondrial Complex IV, enhancing ATP production and supporting energy-dependent cellular processes. PBM also activates light-sensitive ion channels, including transient receptor potential (TRP) channels, increasing intracellular Ca^{2+} levels. This calcium acts as a secondary messenger to activate transcription factors such as NF-κB, AP-1, and CREB, which regulate genes involved in proliferation, differentiation, inflammation, and apoptosis (8, 9). Through these pathways, PBM can modulate immune responses, promote tissue repair, and inhibit tumor growth.

Recent studies suggest that PBM can enhance anti-tumor immunity by promoting NK cell activity, dendritic cell maturation, and cytokine production12. These immunomodulatory effects indicate that PBM could serve as an adjuvant to NK cell-based therapies, improving NK cell function, tumor infiltration, and overcoming immune suppression within the tumor microenvironment.

Mechanistically, PBM enhances NK cell activity through interconnected pathways by increasing ATP production and generating controlled ROS levels, which activate transcription factors including NF-κB and AP-1. This activation leads to elevated cytokine secretion and increased NK cell cytotoxicity. PBM has also been shown to enhance the production of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) and to facilitate interactions between NK cells and antigen-presenting cells, thereby supporting NK cell survival, trafficking, and antitumor activity in vivo.

Given the increasing evidence supporting both PBM-mediated immunomodulation and NK cell-based immunotherapy, their combined application represents a promising therapeutic strategy. Therefore, the objective of this study was to investigate whether PBM could enhance NK cell therapy in a CRC xenograft mouse model and to evaluate the expression of RASSF1A, a gene associated with CRC progression and treatment response. To our knowledge, this is the first in vivo evaluation of PBM-NK combination therapy in CRC.

Materials and Methods

Cells

The Human colon cancer cell line HT-29 was obtained from the Genetic and Biological Resources Center of Iran. The cells were cultured in DMEM medium (Biosera, France) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin (Invitrogen, USA), and 100 µg/mL streptomycin (Invitrogen, USA) in a humidified atmosphere containing 5% CO_2.

NK cells were enriched using standard procedures, and their purity was assessed by flow cytometry based on $CD3^-$ and $CD56^+$ surface markers. Specifically, peripheral blood mononuclear cells (PBMCs) were isolated, and the NK cell population was identified and gated as $CD3^-CD56^+$ using flow cytometric analysis. The cells were then cultured in a basal medium consisting of NK MACS GMP Medium (Miltenyi Biotec, USA), supplemented with 5% (v/v) human AB serum (Valley Biomedical), 500 IU/mL IL-2 (Miltenyi Biotec), and IL-15 (Miltenyi Biotec, USA).

Cytotoxicity of NK Cells Against the HT-29 Cell Line

NK cells (effectors) were co-cultured with HT-29 cells (targets) at effector-to-target (E:T) ratios of 1:1, 3:1, and 5:1 and incubated at 37 °C for 4 hours. Subsequently, the cells were incubated with MTT solution (Roche, Switzerland) for 3 hours at 37 °C in a 5% CO_2 atmosphere. The percentage of lysed cells was determined by measuring the absorbance at 570 nm.

Xenograft CRC Mouse Model

Twelve eight-week-old nude mice were prepared and housed under standard conditions with unlimited access to food and water. HT-29 cells were injected subcutaneously into the back of neck of the mice.

Tumor growth was monitored, and treatment with NK cells was initiated when the tumor volume reached a palpable size. The mice were divided into four groups to evaluate the efficacy of therapy with NK cells and PBM. The groups were treated with PBS, NK cells, PBM, or a combination of NK cells and PBM (Table 1). Tumor dimensions were measured using a Vernier caliper, and tumor volumes were calculated using the π/6 (length × width × height) formula. Tumor volume was measured every other day using a digital caliper, starting from Day 0 (the first day of treatment) until the end of the experiment. Measurements continued every other day for 14 days post-treatment, after which the mice were euthanized for tissue collection and histological analysis.

PBM Protocol and NK Cell Administration

In the PBM group, we used a dedicated PBM system, emitting light at a wavelength of 808 nm. This wavelength has been extensively documented in prior studies for its effectiveness in PBM therapies. The choice of 808 nm is supported by evidence indicating that it penetrates tissues effectively while minimizing thermal damage, making it ideal for therapeutic applications in oncology. Previous research has demonstrated that this wavelength can modulate cellular metabolism, enhance mitochondrial function, and induce apoptosis in cancer cells, thereby improving therapeutic outcomes in various malignancies (10-12).

For the treatment protocol, PBM light was applied directly to the tumor area with a 5 mm margin to ensure targeted irradiation. The treatment was administered in a circular manner for 2 minutes. This localized approach was designed to maximize the effects of PBM on the tumor microenvironment while minimizing exposure to surrounding healthy tissue. PBM was performed as a single session of 808 nm PBM irradiation, applied 7 days after NK cell administration. The PBM exposure lasted 2 minutes, delivering a total energy of 5 J to the tumor site using a low-level PBM device. In the NK cell therapy group, each mouse received an intravenous injection of 15×10^6 NK cells via the tail vein. In the combination group, PBM therapy was performed one week after NK cell infusion. This timing was strategically chosen to assess the impact of PBM therapy on enhancing NK cell activity and their infiltration into tumor tissue.

Histopathology and Immunohistochemistry Assays

Prior to euthanasia, animals were anesthetized with ketamine and xylazine and then euthanized using CO_2. Following euthanasia, the tumors were dissected, and tumor sections were prepared and stained (13). For each animal, one histological section was prepared and analyzed. A total of three animals per group were included, resulting in three sections per group. Histopathological responses to the treatment included tumor necrosis and nuclear polymorphism (5). Nuclear polymorphism was scored according to established histopathological criteria, considering variability in nuclear size, shape, chromatin pattern, and the presence of nucleoli. A scoring system from 0 to 3 was used, and the mitotic count (the number of mitoses in 10 high-power fields [10 HPF]) was assessed using hematoxylin and eosin (H&E) staining, as previously described by Meuten and colleagues (14). Additionally, Immunohistochemistry was employed to demonstrate the presence of ki-67 and caspase-3 in on the tumor tissue sections as markers of tumor proliferation.

Tumor tissue sections were stained for Ki-67 (a nuclear proliferation marker) and Caspase-3 (a cytoplasmic apoptosis marker). For quantification, five high-power fields (HPFs) per section were selected, and the percentage of positively stained cells was calculated in each field. Ki-67 expression was evaluated by determining the percentage of tumor cells exhibiting nuclear staining, whereas Caspase-3 expression was evaluated based on cytoplasmic staining in viable tumor cells.

Real-time PCR Test

RNA was extracted from the isolated tissue using the Pars Toss kit. Following DNase treatment, cDNA synthesis was performed on the treated RNA using the Takara cDNA synthesis kit. Expression analysis of the RASSF1A gene was conducted using the SYBR Green method on a LightCycler 96 thermocycler (Roche, Germany). The sequences of the specific primers used are shown in Table 2.

Table 1: Treatment programs in the studied groups of the xenograft CRC mouse model.

	Group (0,1,2,3)	Treatment description
0	Control	Untreated (PBS only)
1	NK	15×10^6 NK cells (IV infusion)
2	Light (PBM)	Light irradiation only
3	NK + Light (PBM)	15×10^6 NK cells (IV infusion) + light irradiation

Table 2: Sequences and optimized conditions of primers used in real-time PCR.

Gene name	Sequence (5′ to 3′)	Thermal profile
RASSF1A	F: GCCTGAGCTCATTGAGCTG R: ACCAGCTGCCGTGTGG	1 cycle: 95 °C (10 min) 40 cycles: 95 °C (30 s)/ 58 °C (30 s)/ 72 °C (30 s)
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA R: GTCATTGATGGCAACAATATCCACT	1 cycle: 95 °C (10 min) 40 cycles: 95 °C (30 s)/ 60 °C (30 s) /72 °C (30 s)

After optimizing the RT-PCR conditions, real-time RT-PCR reactions were performed in duplicate for each gene. The GAPDH gene cDNA was used as an internal control and was defined in the instrument software. PCR reactions were prepared in a total volume of 20 µL, containing 0.5 µL of each primer (10 pmol/ µL), 2 µL of cDNA, and 10 µL of SYBR Premix Ex Taq II (TaKaRa Bio Inc., Japan).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version X; GraphPad Software, San Diego, CA, USA). Data distribution was assessed for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to compare differences between groups. All data are presented as mean±standard deviation (SD). A p-value less than 0.05 was considered statistically significant.

Ethics Statement

The study protocol involving the use of human peripheral blood samples for NK cell isolation was approved by the Research Ethics Committee of the University of Sistan and Baluchestan (Approval ID: IR.USB.REC.1399.031). All procedures involving human participants were conducted in accordance with the ethical standards of the institutional committee and the Declaration of Helsinki. Written informed consent was obtained from all donors prior to sample collection.

All animal experiments were conducted under a protocol approved by the same ethics committee and complied with international guidelines for the care and use of laboratory animals. Efforts were made to minimize animal suffering. At the end of the experimental period, mice were euthanized by CO_2 asphyxiation following ketamine/xylazine anesthesia to ensure minimal distress before tissue collection.

Results

Ex Vivo Expanded NK Cells Effects on HT-29 Cell Line

The effects of NK cells (effectors) on the HT-29 cell line (targets) were assessed through co-culture at various effector-to-target (E:T) ratios, followed by an MTT assay. The MTT assay measures short-term cell viability and metabolic activity, providing an indication of NK cell–mediated cytotoxicity, rather than complete tumor eradication. The results demonstrated a significant reduction in HT-29 cell viability across all tested E:T ratios (Figure 1). The greatest reduction was observed at an E:T ratio of 5:1 ($P<0.0001$), although this was not statistically significant compared to the 3:1 ratio.

In vivo, the antitumor effect of NK cells was confirmed by a significant reduction in tumor size in treated mice, supporting the relevance of these cytotoxic effects.

Impact of NK Cells, PBM, and Combination Therapy on Morphometric Growth Properties of Colorectal Tumors

The morphometric characteristics of xenograft tumor models were evaluated following individual and combined therapies involving PBM and NK cell therapy. Both treatment modalities, whether administered alone or in combination, led to a reduction in relative tumor volume (Figure 2). The most pronounced suppression of tumor growth was observed in the NK + PBM group, while the PBM-only and NK-only groups exhibited moderate effects, respectively.

Impact of NK Cells, PBM, and Combination Therapy on Tumor Proliferation

The effects of treatment interventions on tumor proliferation were evaluated by assessing the mitotic count in histopathological samples. Compared to the control group, a reduction in mitotic activity was observed in all treatment groups, including NK cells, NK cells combined with PBM (NK + PBM), and PBM alone. However, these decreases did not reach statistical significance (Figure 3). The lowest mitotic count was recorded in the NK + PBM group, with an average of 25 cells per 10 high-power fields (HPF), compared to 32 cells per 10 HPF in the control group. Although this trend suggests a potential anti-proliferative effect of the combined therapy, further studies with larger sample sizes are required to confirm its statistical and biological significance.

Proliferation and Apoptosis Ratios in Tumor Cells Evaluated by Ki-67 Expression

The proliferative activity of tumor cells was assessed by measuring Ki-67 expression using immunohistochemistry (IHC). Ki-67-positive cells were defined as those showing nuclear staining in

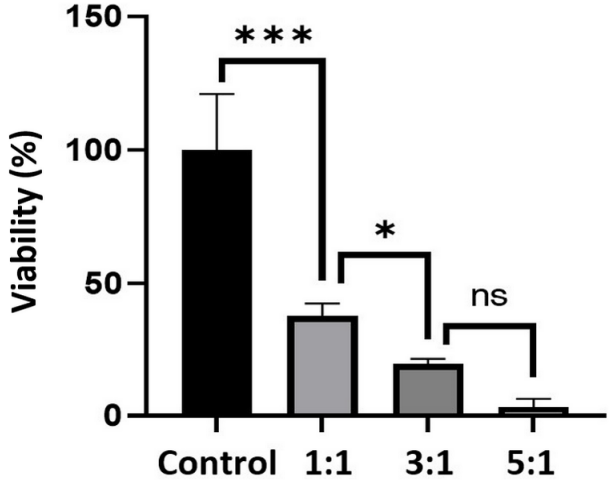

Figure 1: The ex vivo-expanded NK cells demonstrated cytotoxicity against the HT-29 cell line.

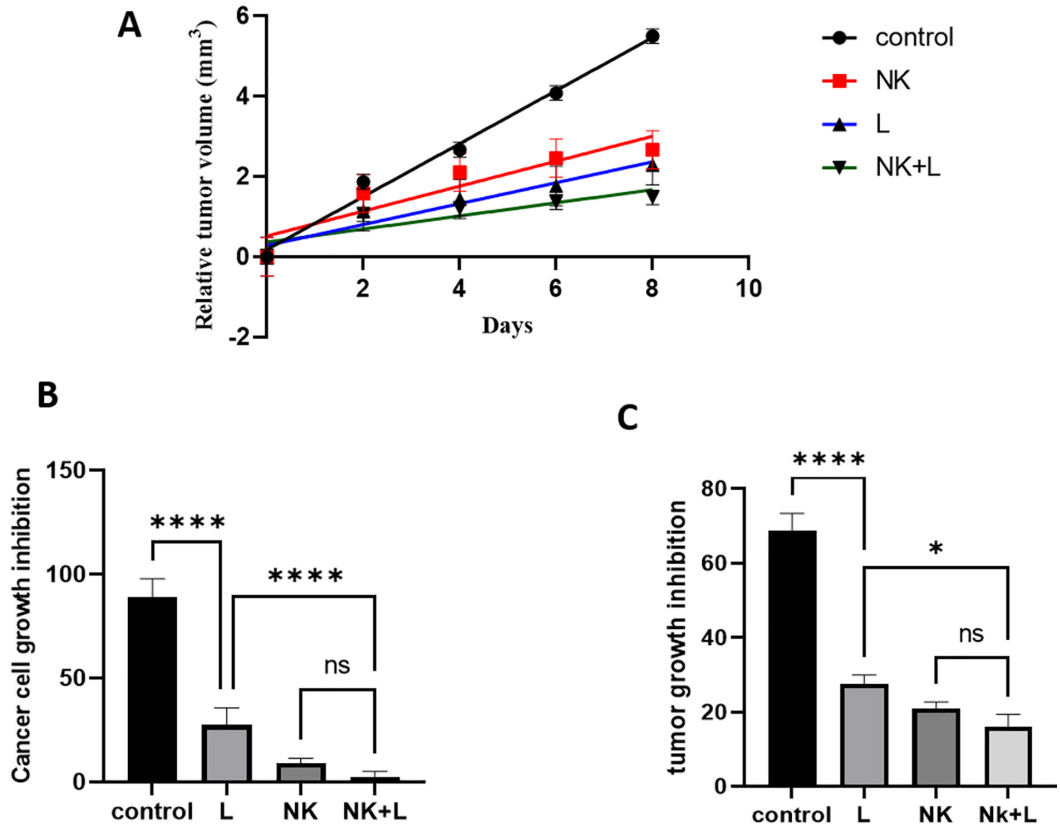

Figure 2: This figure illustrates the effects of NK cell therapy, photobiomodulation (PBM), and combination therapy on the morphometric growth characteristics of colorectal tumors.

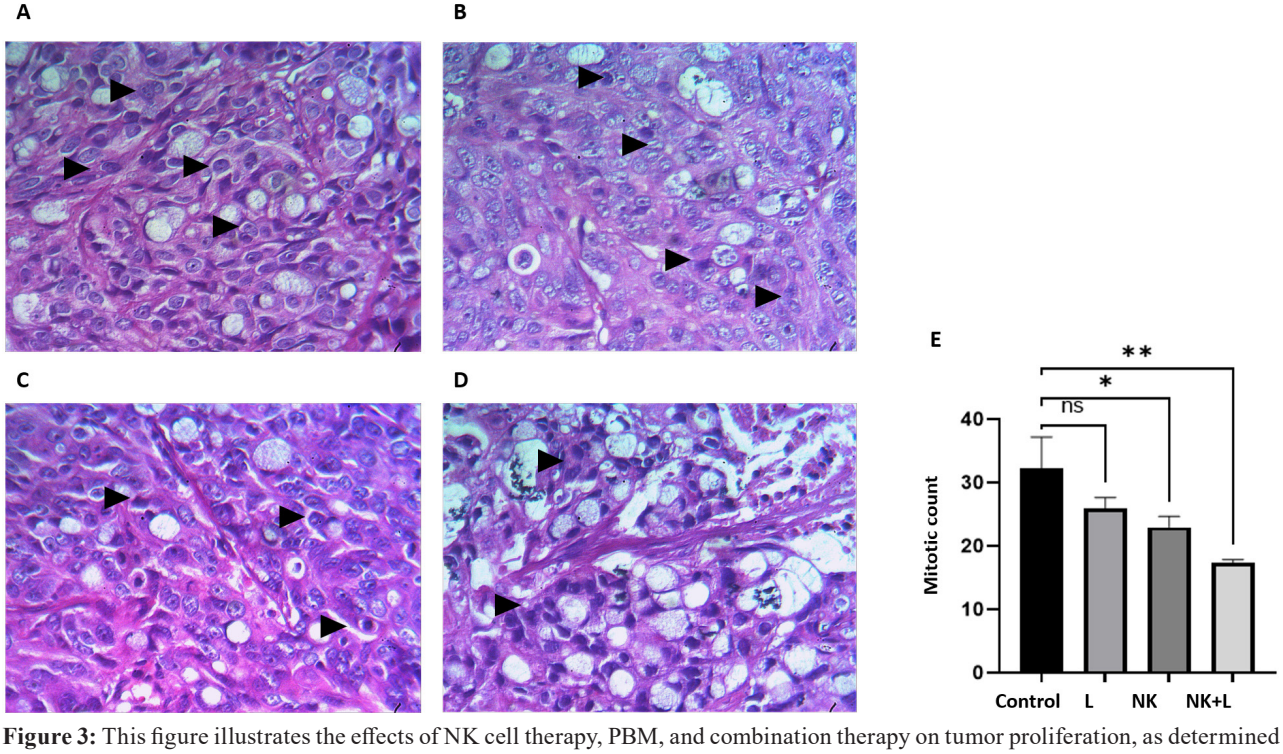

Figure 3: This figure illustrates the effects of NK cell therapy, PBM, and combination therapy on tumor proliferation, as determined by the mitotic count observed in the histopathological analysis.

viable tumor cells, indicating proliferative activity. The control group exhibited a Ki-67 expression rate of 95%. In comparison, the PBM-treated group showed a modest reduction to 86%, which was not statistically significant. In contrast, a significant decrease in Ki-67 expression was observed in both the NK cell group (72%) and the NK + PBM combination group (70%) (P=0.018), indicating that these interventions effectively suppressed tumor cell proliferation (Figure 4).

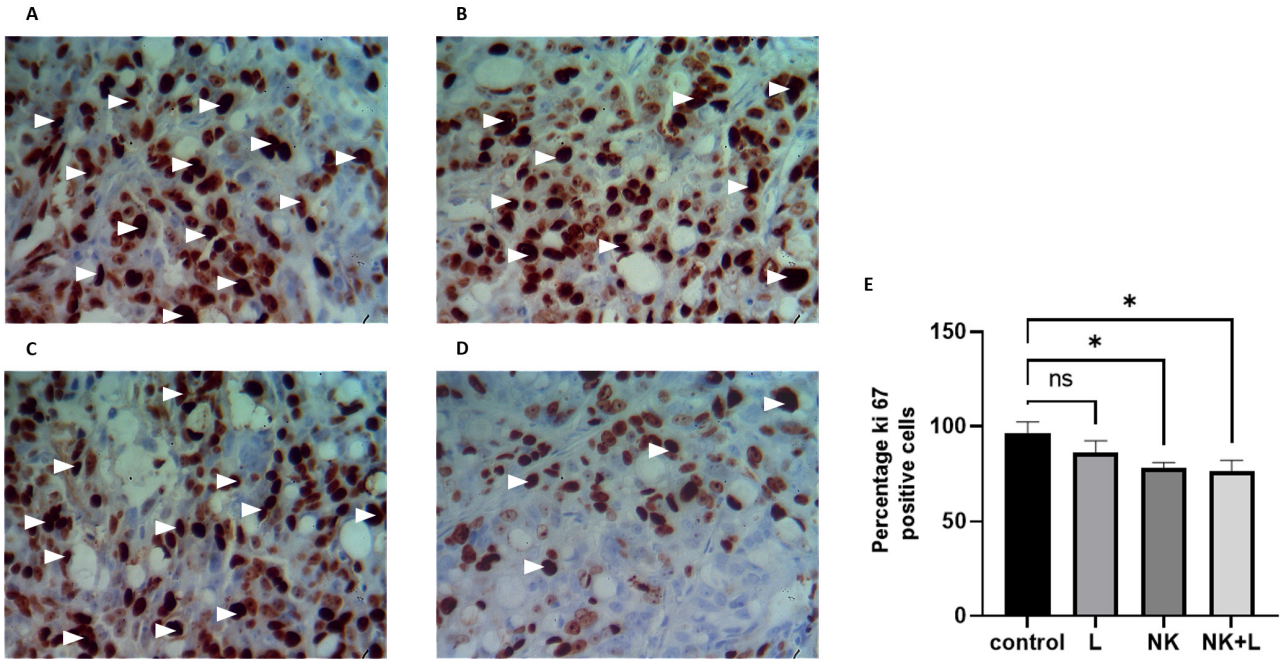

Figure 4: Evaluation of tumor cell proliferation based on Ki-67 expression.

Impact of NK Cells, PBM, and Combination Therapy on RASSF1A Expression Levels in CRC Cells

In CRC tumor cells, the expression levels of RASSF1A play a critical role in tumor behavior and patient prognosis. RASSF1A, a well-established tumor suppressor, is frequently downregulated in CRC, and its loss is associated with increased tumor growth and metastatic potential. Methylation of the RASSF1A promoter has been linked to poor outcomes and resistance to chemotherapy, underscoring its potential as a prognostic biomarker. Notably, the expression level of RASSF1A increased 1-fold in the PBM treatment group and 1.2-fold in the combined treatment group, highlighting the efficacy of these interventions in modulating this tumor suppressor pathway.

The diagnostic performance of RASSF1A expression was further evaluated using Receiver Operating Characteristic (ROC) curve analysis. As shown in Figure 5, the ROC curve yielded an area under the curve (AUC) of 0.66, indicating a moderate ability to discriminate between CRC patients and healthy controls. The optimal cut-off value was determined to be 48, providing the best balance between sensitivity and specificity according to Youden's index. At this threshold, RASSF1A expression demonstrated measurable but limited accuracy in differentiating the two groups.

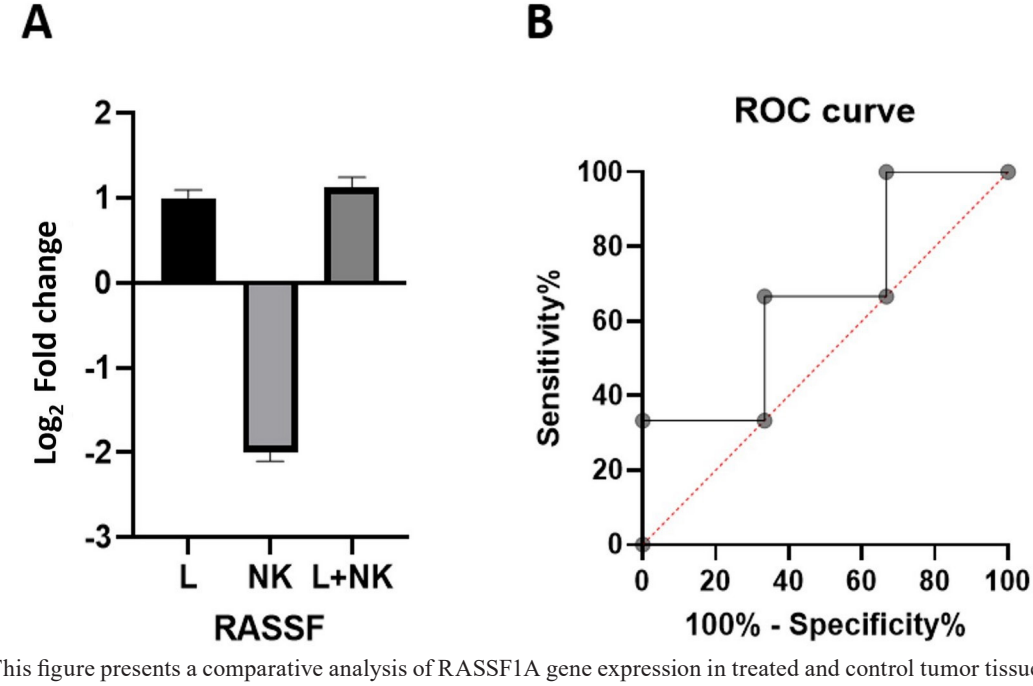

Figure 5: This figure presents a comparative analysis of RASSF1A gene expression in treated and control tumor tissues, along with the corresponding ROC curve.

Discussion

CRC remains one of the most prevalent and deadly malignancies worldwide, despite advances in screening, surgery, and chemotherapeutic strategies. The high recurrence rate and resistance to therapy underscore the need for novel and more effective treatment approaches. Given these challenges, immunotherapy utilizing NK cells represents a promising therapeutic avenue. These innate lymphoid cells provide a critical first line of defense against malignancies, capable of inducing target cell apoptosis without prior antigenic exposure, thereby offering a potent and rapid antitumor response (6). Enhancing NK cell activity through adjuvant treatments may improve their clinical efficacy in solid tumors, including CRC.

In the present study, we evaluated the therapeutic potential of combining PBM therapy with adoptive NK cell transfer in a xenograft model of HT-29 CRC. Our results demonstrate that although NK cells alone significantly reduced tumor volume, their antitumor effect was further enhanced when combined with PBM at 808 nm. This finding suggests a possible synergistic interaction between PBM and NK cell activity.

Previous studies have reported that PBM can influence tumor biology by activating mitochondria, which leads to increased ROS production and apoptosis in cancer cells (15). Moreover, PBM has been shown to modulate immune responses. For example, Zhao et al. (2024) found that PBM increased IFN-γ production and enhanced NK cell cytotoxicity in a tumor-bearing mouse model (8). These findings support our observation that PBM may act as a biological modulator, enhancing NK cell function within the tumor microenvironment.

Importantly, in our previous work utilizing a wavelength-specific PBM system (650 nm and 870 nm) developed by Albert Lazar, we demonstrated that different PBM wavelengths exert distinct biological effects on CRC cells (9).

While the combination of NK cells and PBM has not been extensively studied, related research supports the benefits of integrating NK cell therapy with other adjuvant strategies. Chen et al. (2020) demonstrated that cetuximab combined with NK cells significantly inhibited tumor growth in CRC xenografts (16). Additionally, the use of bevacizumab enhanced NK cell infiltration and antitumor activity in LoVo tumors (17). These findings align with our results, showing that the combination therapy group exhibited significantly increased apoptosis and decreased proliferation, as demonstrated by immunohistochemical staining for Caspase-3 and Ki-67.

Furthermore, we investigated the impact of PBM on gene expression in CRC, focusing on RASSF1A, which is associated with colorectal tumor progression. Several studies have demonstrated that PBM can alter gene expression profiles in cancer cells, including the modulation of transcription factors and tumor suppressor genes (18). In the present study, we analyzed the diagnostic potential of RASSF gene expression in patients who underwent PBM-based therapy. Using ROC curve analysis, RASSF demonstrated an AUC of 0.66 with an optimal cut-off value of 48, suggesting a moderate ability to discriminate between cancer patients and healthy controls. Although the discriminatory power was insufficient to establish RASSF as a standalone diagnostic marker, these findings highlight its potential as a supportive biomarker when used in combination with other molecular or clinical parameters.

Importantly, the observation that RASSF expression demonstrated measurable discriminatory performance even after PBM treatment suggests that this gene may also serve as a molecular indicator of therapeutic response. This raises the possibility of integrating RASSF analysis into treatment monitoring strategies, particularly in contexts where PBM or other targeted therapies are applied.

However, several limitations should be acknowledged. First, the AUC value of 0.66 indicates only moderate diagnostic accuracy, suggesting that RASSF alone is insufficient for reliable clinical application. Second, the relatively small sample size may limit the robustness and generalizability of the findings. Additionally, because the ROC analysis focused on a single gene, integrating RASSF with other molecular biomarkers or clinical parameters could substantially improve diagnostic and prognostic power. Our study also included a limited number of animals per group, lacked long-term follow-up, and did not incorporate mechanistic in vitro assays to clarify the specific pathways through which PBM influences NK cell activity.

Future studies should include larger and more diverse cohorts, employ multi-marker panels to enhance diagnostic accuracy, and perform comprehensive mechanistic experiments, such as signaling pathway analyses, cytokine profiling, and co-culture assays, to elucidate how PBM modulates NK cell function. Additionally, long-term in vivo monitoring and evaluation of combination strategies across various tumor models would further enhance the translational potential of this approach.

In summary, our study provides preclinical evidence that PBM can enhance the efficacy of NK cell-based immunotherapy in CRC. This combination strategy represents a novel therapeutic approach that warrants further investigation to elucidate the underlying mechanisms and assess its clinical relevance.

Conclusion

In conclusion, our findings suggest that RASSF has potential as a complementary biomarker in cancer patients undergoing PBM therapy. Although its moderate AUC value limits its utility as a standalone diagnostic marker, future studies involving larger cohorts and multi-marker panels may help establish

a more precise role for RASSF in both cancer detection and therapeutic monitoring**.** Our results also indicate that the combination of PBM and NK cell therapy enhances anti-tumor effects in a CRC xenograft model more effectively than either treatment alone. This combinatorial approach was associated with increased apoptosis and reduced tumor cell proliferation. While these findings are encouraging, they are based on preclinical data and should be interpreted with caution. The underlying mechanisms of this potential synergy remain to be elucidated. Therefore, further studies are warranted to confirm the reproducibility of these effects, clarify the molecular mechanisms involved, and evaluate the therapeutic potential of NK cell-based PBM immunotherapy in other cancer models and eventual clinical applications.

Authors' Contribution

Najme Malekzadeh Gonabadi conceptualized and supervised the study, and coordinated PBM treatment and in vivo experiments. NK cells were prepared by Dr. Ahamd Vand. The costs of the animal experiments were supported by Dr. Shojaei. Najme Malekzadeh Gonabadi conducted the experiments, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest: No conflict of interest.

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