The Effects of Sorafenib and Natural Killer Cell Co-injection in Combinational Treatment of Hepatocellular Carcinoma; In Vivo

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Research Article

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Abstract

Background: Natural killer cells (NKC) and Sorafenib (Sor) are two important agents for treatment of Hepatocellular Carcinoma (HCC). Over the last decade, the interaction of Sor and NKC against HCC tumors has been very challenging. This study aimed to assess the efficacy of combination therapy of NKC plus Sor for HCC *in vivo*.

Methods: Subcutaneous xenograft models of HCC were established in nude mice. For safety assessment of the treatment, the kidney and liver functions were analyzed. Paraffin embedded tumor sections were histopathologically studied and IHC tests were done to evaluate the angiogenesis (CD34) and proliferation (Ki67) indexes. The TUNEL assay was performed to identify tumor apoptosis. Serum levels of TNF-α and IFN-γ were measured by ELISA assay and expression levels of major inflammatory cytokines and cytoplasmic granules in xenograft HCC tumors were quantified by using real-time PCR.

Results: Combination therapy with NKC and Sor significantly inhibited necrosis and apoptosis in tumor cells and increased angiogenesis and proliferation of HCC cells compared to monotherapy of NKC or Sor alone. The serum levels of TNF-α, IFN-γ as well as the expression levels of TNF-α, IFN-γ, ILs-1, 6 and 10, granzyme B and perforin in the xenograft HCC tumor tissues of mouse treated with both NKC and Sor were significantly decreased than those detected in xenograft HCC groups treated with NKC or Sor alone.

Conclusion: Combination therapy of the specific dosage of NKC and Sor cannot inhibit the HCC xenograft growth rate through a synergistic effect.

All experimental procedures were performed according to the National Institutes of Health (NIH) guide for the care and use of Laboratory animals and were approved by the Institutional Ethical Committee of Tehran University (IECTU) of Medical Sciences (IR.TUMS.VCR.REC.1397.181)

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and it is the third leading cause of cancer deaths worldwide (1). Up to now, numerous therapeutic approaches, including liver transplantation, surgical resection, ablation, **chemotherapy and radiation are used** for treatment of HCC (2).

Sorafenib (Sor) belong to multikinase inhibitor drugs and has ability for inhibition of RAF/MEK/ERK pathway and some tyrosine kinases receptors (TKRs), such as platelet derived growth factor receptors (PDGFR-β), vascular endothelial growth factor receptor (VEGFR-2, -3), Flt-3, and c-KIT. It's well documented that it harnesses cell proliferation, tumor growth, and angiogenesis and induces apoptosis in tumoral cells, as well (3). Although, this therapeutic agent offers some survival benefits in HCC patients, its median survival rate for advanced HCC is still limited. In addition, in some patients, who have been underwent to medication, drug resistant was reported. Therefore, developing of effective therapeutic approaches are crucial for treatment of HHC (4, 5).

In recent years, scientist pay more attention to combination therapy as new therapeutic approach in HCC. For better suppression of advanced HCC, many efforts have been recently made via combination therapy include Sor and other agents (2, 6-10).

Over recent decade, immune cell therapy by using NK cells has been highly considered for HCC. This new method has high therapeutic potential for advanced HCC due to its critical role in innate immune system of liver and its anti-viral and anti-tumor properties, as well. Moreover, when using NK cells for HCC, there is not a risk for graft-versus-host disease (GVHD) via its receptors inhibition activity in the presence of surface major histocompatibility complex (MHC) class I (11-13). Despite these benefits, its frequency and cytolytic activity might be impaired in the progressive stage of HCC (14). In this regard, combination of immune cell therapy accombined with other approaches seems necessary for improving the HCC treatment. So far, many studies have been conducted to improve dysfunction or exhaustion of NK cells using various strategies, including chemoimmunotherapy, transplantation and genetic manipulation of NK cells, preparation of NK cell lines, mAb and cytokine therapy (2, 7, 15).

One of the most attractive and challenging approaches was developed over the last decade is the combination of Sor and NK cells against HCC. Various study findings demonstrated that there are two different outcomes; in some studies was reported the synergic effect (16-19) and in the other ones was reported the inhibitory effect (20-24). Eventually, the paradoxical effects of Sor on NK cell effector functions, which are in relation with dose and time, are quite considerable (25). There is a narrow therapeutic window between the immune cell activation and the appropriate anti-tumor effects of this drug. Therefore, in this study we aim to investigate the *in vivo* therapeutic efficacy and safety of simultaneous injection of Sor and adoptive NK cells in comparison to individually administration in xenograft mouse model bearing of HCC.

Materials And Methods

Reagents

Sorafenib (Nexavar, Bayer, 43-9006) was purchased from the American LC LAB Company and was dissolved in 12.5% ethanol, 12.5% Cremophor, and 75% water (1:1:6) (20). The dissolved Sorafenib was used at the concentration of 30 mg/kg/day in the animal model, which is in accordance with the range of respective dosage used for human (400 mg, twice daily) (26). Antibodies against CD56 (PE, EXBIO, Czech Republic) and CD3 (FITC, Beckman Coulter, US) were used for flow cytometric analysis. Human recombinant interleukin-2 (specific activity of greater than or equal to 5.7 x 10e6 Units/mg) was purchased from eBioscience (US).

Cell Lines and Animals

Human HCC cell line (HepG2) was purchased from the Iranian Biological Resource Center and cultured in high glucose DMEM media (Gibco D5796, USA), supplemented with 10% fetal bovine serum (Gibco,USA) and penicillin and streptomycin (100 µg/ml). The cells were then incubated in a 37°C, 95%

humidified atmosphere containing 5% CO_2. Cells at second or third passages were used for xenograft injection to immunodeficient mice and creation of a suitable HepG2 mouse model. Male athymic C57BL/6 nu/nu mice (mean weight: 20 g; age: 4 -6 weeks) were purchased from the Pasteur Institute of Iran. (27). All experimental procedures were performed according to the National Institutes of Health (NIH) guide for the care and use of Laboratory animals and were approved by the Institutional Ethical Committee of Tehran University (IECTU) of Medical Sciences (IR.TUMS.VCR.REC.1397.181) (28).

Isolation, expansion and activation of NK Cells

Primary NK cell was collected from buffy coats of healthy donors under the approval of the IECTU of Medical Sciences and then expanded and activated under specific condition as detailed in our previous article (29). Briefly, Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-Hypaque (GE Healthcare, **GE17-5442-02**Sigma, and Sweden). NK cell was isolated from PB using the NK Cell Isolation Kit and MACS columns (Miltenyi Biotec, Germany) by negative selection kits, according to the manufacturer's procedures. The purified NK cells were cultured in SCGM medium (CellGenix, Freiburg, Germany), containing anti-CD3 Antibody (OKT3) (Cytomatin Gene, Iran) (10 ng/ml), penicillin (100 IU/mL), 10% FBS, and streptomycin (100 mg/mL) with irradiated autologous PBMCs as feeder layer. The activation process was performed by human recombinant interleukin-2 (hrIL-2) (eBioscience™, US) (1,000 IU/mL) and hrIL-15 (eBioscience™, US) (10 ng/ml) (30). Purity of the isolated and expanded human $CD3^-$, $CD56^+$ NK cells were determined using Attune NxT acoustic focusing flow cytometer. On average, purity of the isolated and expanded NK cells were more than 95% in all experiments.

HepG2 xenograft model and treatment

To implant heterotopic HepG2 tumors, as described in our previous studies (27, 31). Briefly, 1×10^7 human HepG2 cells suspended in 200 µl of a 1:1 ratio of serum-free medium and Matrigel (Corning, product number: 354230, USA) were injected subcutaneously into the two flanks of each animal. Tumor formation was monitored twice a week until day 40 p.i of HepG2 cells. Tumor volume was calculated with the means of Vernier calipers using a standard formula (length × $width^2$ × 0.5) followed by drawing the growth curves (32). When the average of tumor volumes approximately reached to 200 mm^3 (12^{th} day), which is equivalent to advanced stage (27), the mice were randomly assigned to four experimental groups (control, Sor, NK cells and Sor plus NK cells) with three mice in each group. Sor (30 mg kg^{-1}, daily) was intraperitoneally (IP) injected (33) while human IL-2 activated NK cells (5×10^6 cells/100µl/mouse) were injected IP (30) into the tumor margins twice with one-week interval. An equal volume of carrier solution was injected in the CG. Four weeks after the onset of treatment, the mice were sacrificed followed by collecting their blood samples and tumor tissues (30, 34). Some of tumor tissues were fast frozen in liquid nitrogen and then stored at −80 °C and the rest tissue was fixed in 10% neutral buffered formalin (NBF) followed by paraffin embedding for IHC analysis.

Analysis of biochemical factors for safety assay

To evaluate the in vivo safety of either of the NKC alone or its combination with Sor, body weight of each mouse as well as its liver and kidney functions were analyzed. The blood sample of each mouse was centrifuged at 1500*g* for 10 min followed by collecting the serum and measuring the levels of various biochemical factors, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and cratinin, using an automated biochemical analyzer (Mindray, Shenzhen, China).

Histopathology study

The mice were euthanized on day 28 post-treatment and the obtained tissues (tumor mass) were fixed in the 10% NBF (PH= 7.26), followed by being processed and embedded in paraffin. Tumor tissue were sectioned at a thickness of 5μm and were stained with heamtoxylin and eosin (H&E). The histological sections were evaluated by two expert pathologists, using light microscopy (Olympus, Japan). Histopathological examination was performed using the Edmondson-Steiner grading system (1954) for HCC. Moreover, any changes, including coagulative necrosis, inflammatory response, hemorrhage, and hyperemia, were comparatively evaluated in tumor sections of different groups.

Immunohistochemistry

Paraffin-embedded tumoral sections (4 μm) were applied for immunohistochemistry (IHC) evaluation of proliferating cell nuclear antigen (Ki67) using a monoclonal primary mouse anti-human Ki67 antibody (Biocare, CA, USA). The proliferative index was determined by counting the number of positive stained cells amongst 100 nuclei (Percentage) which were randomly counted from five high magnification microscopic fields (200× magnification) using the Image-Pro Plus®V.6 software (Media Cybernetics, Inc,Silver Spring, USA).

To assess the angiogenesis index (AI) of HCC tumor, the sections were stained using a monoclonal mouse anti-human CD34 antibody (Biocare, CA, USA). The AI was defined by counting the number of positive stained cells for CD34 in five high magnification microscopic fields (200× magnification). The results of angiogenesis were reported as the mean number of vessels ± SEM. The negative control sections were prepared by omitting the primary antibody for Ki67 and CD34.

TUNEL assay

TUNEL Assay Kit (Abcam, UK) was used to detect apoptotic DNA fragmentation. To compare the anti-tumor efficacy of each treatment via enhancing the number of apoptotic tumor cells *in vivo,* the tumor tissue sections were stained using TUNEL according to manufacturer's procedure and were visualized using a fluorescence microscope . The TUNEL positive cells were counted in three microscopic fields per section and reported as the mean percentage of the total apoptotic index.

Determination of serum TNF-α and IFN-γ

The serum sample was harvested from each mouse and analyzed by ELISA kits to detect the contents of mouse interferon gamma (IFNG) (ab100689, Abcam, UK) and Mouse TNF alpha (ab208348, Abcam, UK).

Quantitative Real-Time PCR assay

The expression levels of intended inflammatory factors, including TNF-α, IFN-γ, ILs 1-6 and 10 and cytoplasmic granules (Perforin and Granzyme-B), were quantified using qRT-PCR. Total RNA was extracted from the treated xenograft HCC tumor tissues using a total RNA extraction kit (Takara, Japan) according to the manufacturer's procedure. Isolated RNA with a 260/280 ratio of ~2 (1µg) was reverse-transcribed to cDNA by using a PrimeScript RT reagent Kit (Takara, Japan). Gene expression was determined by ABI-7000 Detection System thermal cycler (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, Japan). The Real-Time PCP reaction mixture was prepared using 10 pM of each of the primers, 100 ng cDNA (2 µl), SYBR Green I Master Mix (2X) (BioFact, Korea) and nuclease-free H2O. The real-time PCR cycle consisted of 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72 °C for 30 seconds and a cycle of melt curve consisted of 95 °C for 15 seconds, 60 ºC for 1 minute and 95 °C for 15 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal comparator in parallel with the control sample. All the qRT-PCR experiments were performed in duplicates. qRT-PCR results were analyzed using the comparative Ct, 2−ΔΔCT, method.

Statistical analysis

The results are reported as the mean ± SEM. Two-way analysis of variance (Two-way ANOVA) was used to compare the mean values obtained from all experimental groups, followed by post-hoc Tukey test. The paired data were analyzed using the 2-tailed paired Student's *t* test. The charts were drawn using GraphPad Prism and the statistical differences were considered to be significant at $P<0.05$ ($p<0.05$ *, $p<0.01$ ** and $p<0.001$ ***).

Results

Purity and *in vivo* safety of NK cells

The purity of human $CD3^-$, $CD56^+$ NK cells, isolated from PB using the NK Cell Isolation Kit and MACS columns (Miltenyi Biotec, Germany), was measured by Attune NxT Acoustic Focusingtometer (Invitrogen, USA). The purity of isolated NK cells was more than 95% (Fig. 1A). To assess in vivo toxicity mediated by NK cell adoptive therapy, alone or in combination with Sor, body weights of all the treated mice were measured. The results of body weights indicated thar there was no significant differences between the NK cells-treated mice and the (control group) CG (Fig.1B). In addition, to analyze therapeutic safety, liver and kidney functions of the mice were evaluated by measuring the serum levels of ALT, AST, BUN, and Cr in all the treatment groups. No significant differences were detected in kidney and liver functions of various treatment groups compared to those of the CG (*p* value> 0.05) (Fig. 1C).

Tumor growth analysis

The therapeutic efficacy of NKC and Sor (individually or combined) against HCC tumor was analyzed by measuring the tumor growth resulted from HepG2 cell xenografts in nude mice. As shown in Fig. 2,

injection of HCC mouse model with Sor (30 mg kg^{-1}, daily) or NK cells (5×10^6/mouse, twice with 1-week interval), individually, significantly inhibited tumor growth compared to the vehicle-treated animals. Half of the tumors treated with NK cells completely disappeared and the results of H&E staining of their tumor sections did not showed any sign of tumor tissue and only the skin tissues were observable (data not shown). However, combination of NK cells plus Sor (NKC & Sor) at the above mentioned dosage induced no synergic effect against HCC tumor growth.

Histopathological study

The H&E-stained tumoral sections obtained from all the experimental groups were histologically evaluated and a solid pattern composed of thick trabeculae compressed into a compact mass was observed in the primary tumors. Grads the tumors of various treatment groups were examined and no differences were found between the groups, according to the Edmondson-Steiner grading system. In all the treatment groups, the tumoral cells were poorly differentiated (high grade, grade III to IV) and the differentiation rates were different in various samples of each group. In histological sections of the CG, numerous paelomorphic tumor bizarre cells (Fig. 3A, arrow heads) and many mitotic figures were observed and the tumoral cells demonstrated severe anisokaryosis and anisocytosis (Fig. 3A, thin arrows). Moreover, various degrees of necrosis were seen in different treated groups of HCC animal models (Fig. 3). The scores of tumor necrosis were the highest in the Sor-treated mice followed by natural killer cells (NKC)-treated animals and there was significant statistical differences between the groups treated individually with either NKC or Sor and the control and combinatinal NKC+Sor treated groups.

Evaluation of tumor proliferation by IHC analysis

The mean percentage of tumor cell (TC) proliferation was determined by counting Ki67-positive cells. As shown in Fig. 4A, the proliferation rates were significantly different between the groups individually treated with either Sor or NKC and the other two groups ($P<0.001$). Monotherapy of the xenograft HCC tumors with either NKC or Sor led to a significant suppression of TC proliferation compared to the CG, however, combination of Sor and NKC did not induce any remarkable effect on the inhibition of TC proliferation.

Evaluation of TC angiogenesis by IHC analysis

Tumor angiogenesis was assessed based on the mean percentage of CD34-positive cells. The density of micro vessels per high-power microscopic field (HPF) in the tumor xenografts of the experimental groups individually treated Sor and NKC was 18.6±1.7 and 37.4±2.1, respectively. However, these values were 48.7±2.2 and 58.7±3.4/HPF in the NKC+Sor and control mice, respectively, which were higher than those of the Sor or NKC-treated groups (Fig. 4B). Treatment of HCC mouse models with Sor resulted in the highest anti-angiogenesis effect followed by NKC treatment and then combination therapy with NKC plus Sor, indicating no synergistic interactions between Sor and NKC treatments against tumor angiogenesis.

Evaluation of TC apoptosis by TUNEL assay

The TUNEL assay was employed to designate whether implementation of Sor and NKC (individually or combined) inhibits the growth of tumor xenografts by inducing apoptosis in the TC, *in vivo*. The proportion of apoptotic-positive cells in both the Sorafenib and NKC groups was significantly higher than that of the NKC+Sor and control groups ($p < 0.01$, Fig. 5). The apoptosis rate was not significantly different between the NKC+Sor and control groups ($p > 0.05$), indicating the lack of an additive effect of NKC and Sor injection (at specific time and dose) against HCC tumor growth through induction of apoptosis.

Determination of serum IFN-γ and TNF-α by ELISA analysis

According to the ELISA finding, the serum levels of IFN-γ and TNF-α in the mice treated with NKC+Sor were significantly lower compared to the control, Sor or NKC treated groups (Fig.6).

Gene expression analysis

The expression levels of most of the target inflammatory cytokines, including TNF-α, IFN-γ, ILs-1, 6, 10 and cytoplasmic granules (perforin and granzyme B), which are produced by activated NK cells were measured in xenograft HCC tumor tissues of all different treatment groups using qRT-PCR analysis. The expression levels of all the above-mentioned genes were significantly up-regulated in the NKC-treated group compared to the CG. However, Sor significantly down-regulated the human TNF-α and IL-1 genes expression levels and up-regulated the expression level of IL-10 gene (p-value < 0.05), but induced no meaningful effect on the other investigated genes expression levels compared to the CG group. The expression levels of all the intended genes were down-regulated in the experimental group treated with the combination of NKC and Sor relative to those detected in the individual NKC or Sor treated groups (Fig. 7), indicating no synergic effect of NKC and Sor against HCC tumors and the inhibitory effect of Sor on the cytokine production and effecter functions of NK cells.

Discussion

Immune-cell therapy of hepatocellular carcinoma with natural killer cells is a remarkable approach that has attracted interest from many researchers in recent years. (11, 35) (12). The anti-tumor effecter function of NK cells is either performed directly through releasing cytoplasmic granules (Perforin and Granzyme) secretion of inflammatory cytokines (TNF-α and IFN-γ), triggering receptor-mediated apoptosis (via FasL or TRAIL) and antibody dependent cellular cytotoxicity by inducing the expression of CD16 antigen in the NK cells, or indirectly via interaction of NK cells with other immune cells (36).

Sorafenib (Sor) is the only FDA approved drug for treatment of patients with advanced HCC. It is an oral multikinase inhibitor which reduces angiogenesis and tumor proliferation via blocking various signaling pathways. Despite the beneficial effects of Sor on improving the overall survival rate and delaying the disease progression, its survival benefit is still limited (median less than one year) and in some cases the tumor regression were observed. Progression of liver diseases lead to a reduction in the frequency and functional impairment of NK cells against HCC tumors (14, 37), therefore, various strategies, which have

been summarized in our previous study, have been reported to be employed to overcome this problem (2). One of the approaches used to address this issue is chemoimmunotherapy by combining NK cells and one chemotherapy drug, such as Sorafenib. Therefore, application of the combination of Sor with other therapeutic agents seems to be beneficial (38, 39) as, can be considered an attractive concept. Therefore, considering the important role of both Sor and NK cells in HCC treatment and the previous antithetical results regaring their interaction against HCC tumors, the present study focused on the combinational immune cell therapy with stimultaneous injection of NK cells and Sorafenib in order to to find their optimal doses in a xenograft HCC nude mice model.

The additive effect of Sor and NK cells on the anti-tumor properties of each other is very controversial. Some studies have shown the synergic effect of Sor and NK cells against hepatocellular carcinoma through various mechanisms (16-18, 40). However, other studies have reported that Sorafenib inhibited the cytotoxic effect of NK cells and induced an immunosuppressive effect through various signaling pathways (3, 20-24). It has been recently shown that the effect of Sor on the NK cell effecter functions is induced in a dose- and time-dependent manner (25). Considering the possible risk of immunosuppression in Sor treated patients, it is essential to find new suitable as an alternative for Sor in combination therapy in order to eliminate the risk of immunosuppression and improve its efficacy against HCC tumors (20).

In our finding, the purity of the isolated Natural Killer cells ($CD3^-$, $CD56^+$) obtained from peripheral blood of healthy donors was more than 95% in all experiment (Fig. 1A). In addition, *in vivo* safety and toxicity of the isolated NK cells, individually and in combination with Sor indicated no significant differences in none of the above-mentioned values between different treatments and the CG (Fig. 1B, 1C). The tumor volumes The efficacy of activated NKC on tumor growth inhibition, as expected in accordance with the results of recently published articles, was significantly higher than that of other treatments compared to the CG(14, 16, 41, 42), so that half of the NKC-treated tumors were completely disappeared and only their skin tissues were remained. In the following, the growth of Sor-treated tumors was significantly inhibited compared to the CG. These findings were in agreement some previous studies (42). However, no significant differences were observed in tumor growth between the NKC plus Sor-treated mouse and the CG, indicating the lack of a synergic effect of NKC and Sor against HCC tumors. The rate of necrosis in the HCC tumor cells indicated that either Sor or NKC was able to significantly induce TC necrosis. However, concurrent therapy with Sorafenib and NKC not only could not enhance the percentage of necrotic TC but also reduced the necrosis rate of the tumor tissues nearly to that of the CG. Immunohistochemical (IHC) analysis demonstrated that the proliferation rate of the HCC tumor cells which were treated with either Sor or NKC was significantly lower than that of the CG($p < 0.001$). These findings were consistent with those of the previous studies which have reported that Sor induced its effects by up-regulation of p53 and suppression of Forkhead box M1 (FoxM1) (43) and over-expression of cytolysis-related molecules, including TRAIL, NKp80,granzyme B, TNF-α and IFN-γ, by NK cells (34). However, co-injection of NKC plus Sor did not induce any significant effect in terms of tumor proliferation compared to the CG, indicating that these two therapeutic agents induce inhibiting effects on each other in terms of their anti-proliferative effects on HCC tumors. The micro vessel densities of all the treatment

groups indicated that Sorafenib dramatically blocked angiogenesis in the HCC tumor compared to the CG ($p<0.001$), as expected according to the findings of the previous studies (44). NK cells also induced a considerable anti-angiogenesis effect against HCC tumors compared to the CG ($p<0.05$). However, combination therapy with NKC plus Sor did not significantly reduce angiogenesis levels of HCC tumors compared to the CG, which might be due to the inhibitory effect of both NKC and Sorafenib on effecter functions of each other, at the prescribed dosages

The results of TUNNEL assay indicated that compared to the CG, both Sor and NKC could significantly inhibit the growth of xenograft HCC in nude mice, individually, via induction of apoptosis ($p<0.001$and $p<0.01$, respectively) (Fig. 5). These findings are in agreement with the findings of previous studies in terms of induction of apoptosis via activation of JNK and Akt/mTOR/p70S6K and signaling pathways and blocking of RAF/MEK/ERK Pathway by Sorafenib (44, 45), as well as aggregation of death receptor-mediated apoptosis proteins and their ligands (FasL and TRAIL) and a caspase-3-dependent pathway by NK cells (46). As shown in Fig. 5, the combination of Sor and NKC not only could not reduce tumor growth *in vivo* via induction of apoptosis, but also inhibits the induced programmed cell death which was triggered by Sor or NKCs, individually. Therefore, it can be concluded that these two therapeutic agents might inhibit the anti-tumoral effects of each other at specific doges and administration time.

The cytotoxicity of NK cells can be evaluated by measuring the cytokines production, such as IFN-γ, TNF-α, ILs- 1, 6 and 10 and cytoplasmic granules (Perforin and Granzyme), which are up-regulated in activated NK cells (2, 36, 47, 48). The serum levels of IFN-γ and TNF-α in different treated groups were measured by ELISA assay kits. As showed in Fig. 6, the serum levels of TNF-α and IFN-γ in the mouse treated with both NKC and Sor were significantly lower than those in the control and other treatments groups, indicating the inhibitory effect of NKC and Sor on the efficacy of each other. As reported by some previous studies, Sor decreased the number of NK cells and impaired IFN-γ production co-cultured with tumor cells, in a dose dependent manner (20-23). However, the there was no significant differences in the serum levels of TNF-α and IFN-γ in the mouse received monotherapy with either NKC or Sor. These findings are consistent with the results of a study conducted by Lei et al., (2016) who have concluded that the plasma levels of cytokines, such as IFN-γ and TNF-α, in HCC patients before and after chemotherapy with Sor showed no significant differences (49). Moreover, another study on RCC patients has reported that Sorafenib did not alter the cytokine responses of peripheral immune effecter cells (50). However, the results of TNF-α and IFN-γ gene expression study in differently treated mouse by qRT-PCR analysis of isolated mRNA from collected xenograft HCC tumor tissues (Fig.7) showed significant alterations. The different results of ELISA and Real-Time PCR analysis from the levels of TNF-α and IFN-γ in serum and tumor tissues, respectively, could be due to either the intra tumoral accumulation of cytokines resulted from local injection of NK cells, or that the increased number of transcripts of these cytokines were not translated to protein when the samples were obtained. The results of ELISA and Real-Time PCR studies indicated that both the mRNA and protein levels of both TNF- α and IFN-γ were significantly decreased in the mice treated with combination of NKC&Sor compared to that in mice treated with NKC or Sor, individually. As illustrated in Fig. 7, injection of NK cells significantly up-regulated the expression levels of TNF-α, IFN-γ, ILs-1, 6, and 10, Perforin and Granzyme-B relative to those in the CG indicating that NK cells are highly

activated and potent in vivo. These findings are in agreement with the findings of previous studies that have demonstrated that activated NK cells promoted the expression of a various cytokines, including TNF-α, IFN-γ, IL-1, IL-10, and cytoplasmic granules (granzyme B and perforin) (47, 48, 51-53).

It has been reported that NK cells have the potential to induce both direct antimicrobial and anti-tumoral effects as well as immune regulatory responses. The results obtained from human and experimental models suggest that NK cells inhibit host immunity during chronic diseases and acute infections by rapidly increasing the expression level of IL-10, which is an anti-inflammatory mediator in limiting immunopathology (54-56). Moreover, the effect of IL-6 on promoting oval cell proliferation, liver regeneration and suppression of HCC by increasing the number of NK cells has been proven (57). These results are consistent with the findings of Cheng et al., (2011) who have suggested that NK cells, through an IFN-γ-dependent mechanism, induced IL-6 production and upregulated expression of Fas in the target HCC cells that led to enhanced susceptibility of HCC cells to NK-mediated cytotoxicity and suppression of liver damage (48). Furthermore, it has been proven that cytokine IL-1 mainly produced by activated NK cells involved in inflammation and tumor development. This inflammatory factor can potently inhibit the growth of hepatocellular carcinoma tumor via induction of T and NK cell activation and stimulation of IFN-γ production by NK cells and (58-60)**.** In addition, these findings are confirmed with the fact that production of some inflammatory factors and cytoplasmic granules, such as IFN-γ and perforin, by intrahepatic NK cells are reduced in patients with advanced stage of liver fibrosis (61). As illustrated in Fig. 7, Sorafenib induced no significant effect on the expression levels of perforin, granzyme, IFN-γ and IL-6 (p value > 0.05), however, it significantly down regulated both IL-1 and TNF-α and induced the expression of IL-10 (p value < 0.05), as have been reported in our previous study (31). Most importantly, the gene expression levels of all the intended gene were the lowest in the mice treated with the combination of NKC & Sor, indicating no synergic effect of NK cells and Sorafenib (at specific dose and time) against HCC tumors. These results confirme the findings of previous studies regarding the inhibitory effects of Sorafenib on NK cells (20-24).

Conclusion

Overall, the results of this study confirmed that concurrent administration of NK cells and Sorafeib cannot inhibit the hepatocellular carcinoma cells growth through a synergistic effect. We assume that the cytotoxicity and cytokine production of NK cells against HCC tumor may be inhibited by Sor, which could not be overcome even with adoptive transfer of IL-2 activated NK cells. Furthermore, the results of combination therapy with NK cells and Sor might indicate the inhibitory effect of NK cells on the efficacy of Sorafenib against proliferation and angiogenesis of tumor cells. Our findings suggest that Sor may not be a preferable chemotherapeutic agent to be used in combination with NK cells for the treatment of hepatocellular carcinoma. However, considering the contradictory effects of Sorafenib on the effector functions of NK cells adopted in a dose and time dependent manner, further studies are required to investigate the effects of various injection doses of Sorafenib and NK cells for combination therapy of HCC.

Declarations

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Authors' contributions

JV designed and guided the study. FH prepared the first draft of the manuscript and JV, JA, SM, SE, IS, TKM, SS, AH, and FH were involved in drafting the manuscript and collecting information. FH, SM and IS prepared the cells and animal model. FH and TKM performed the qRT-PCR analysis. All authors read and approved the final version of the manuscript. SS revised final version of the manuscript.

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Authors' information

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Figures

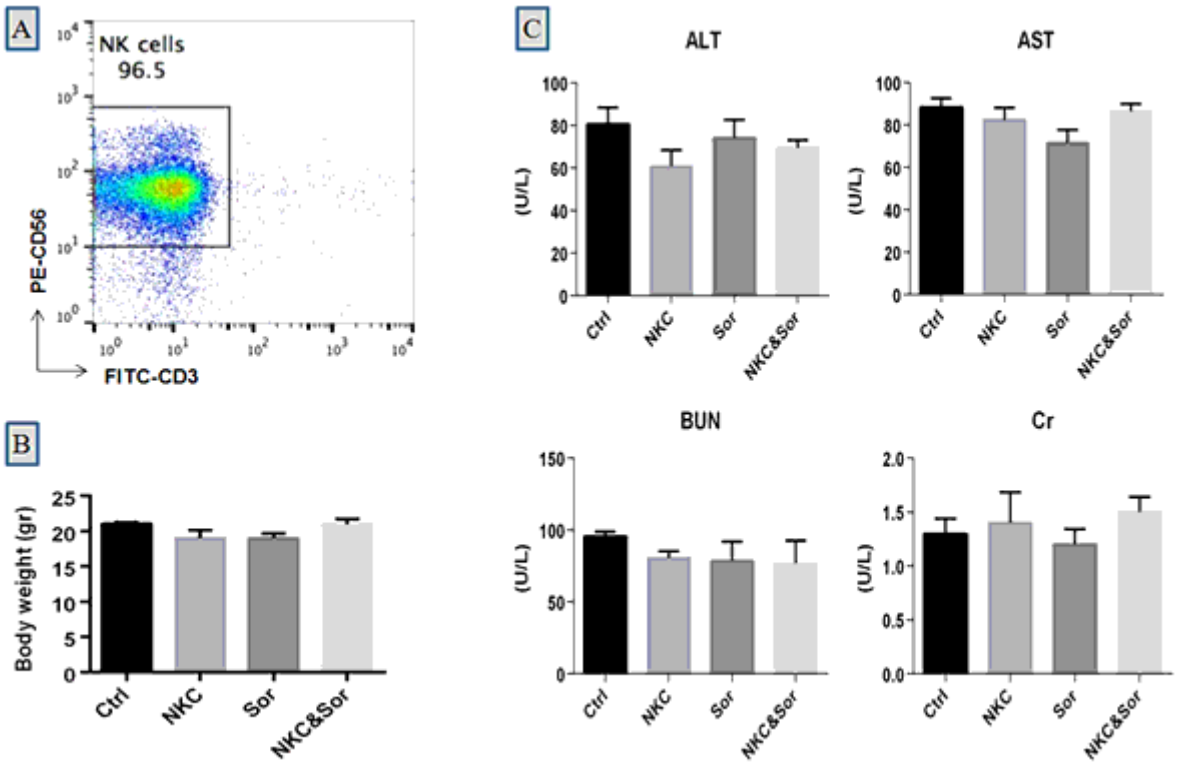

Figure 1

(A) The flow cytometry assay of isolated NK cells from buffy coat of healthy donor by PE-CD56 and FITC-CD3 markers, indicated more than 95% purity of NK cells. (B) Body weights and (C) The serum levels of hepatic and lung biochemical factors (ALT, AST and BUN, Cr respectively) in different treated mouse models of HCC. Statistical analysis did not show any significant differences between those elements of all groups compared Ctrl, indicating no signs of toxicity and liver or kidney dysfunction. The data are expressed as the mean ± SEM. Ctrl: control, Sor: sorafenib, NKC: natural killer cells, ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; Cr: creatinine, SEM: standard error of the mean.

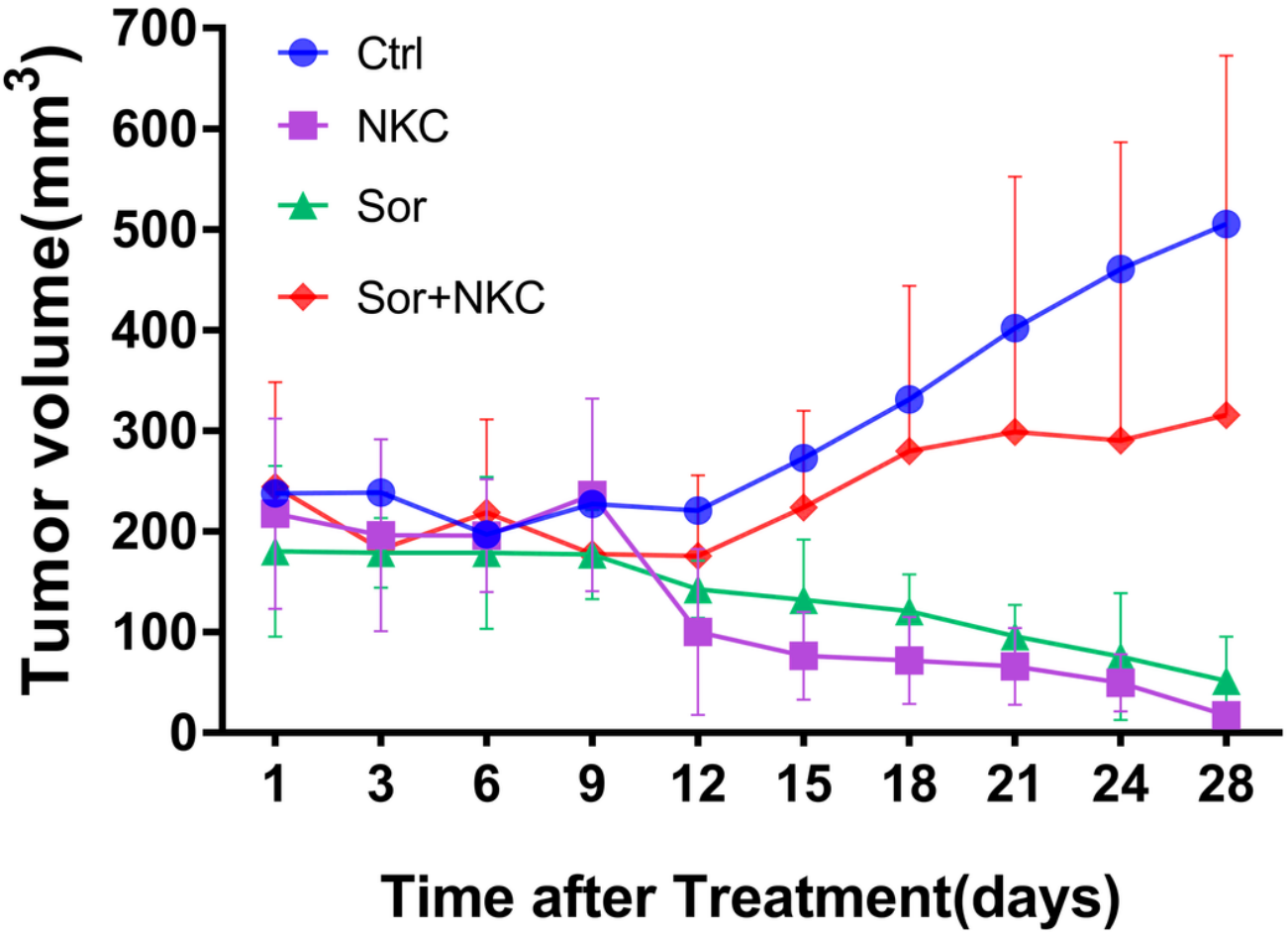

Figure 2

A. Tumor growth of hepatocellular carcinoma xenografts in nude mice in different treated groups since the first day until 28th day after start treatment. Control (Ctrl), Sorafenib (Sor; 30 mg/kg per day), Human Natural Killer cells (NKC; 5 × 106 cells/mouse, two times a week apart), or combination of NKC and Sorafenib (NKC & Sor) (n = 4 tumors each group).

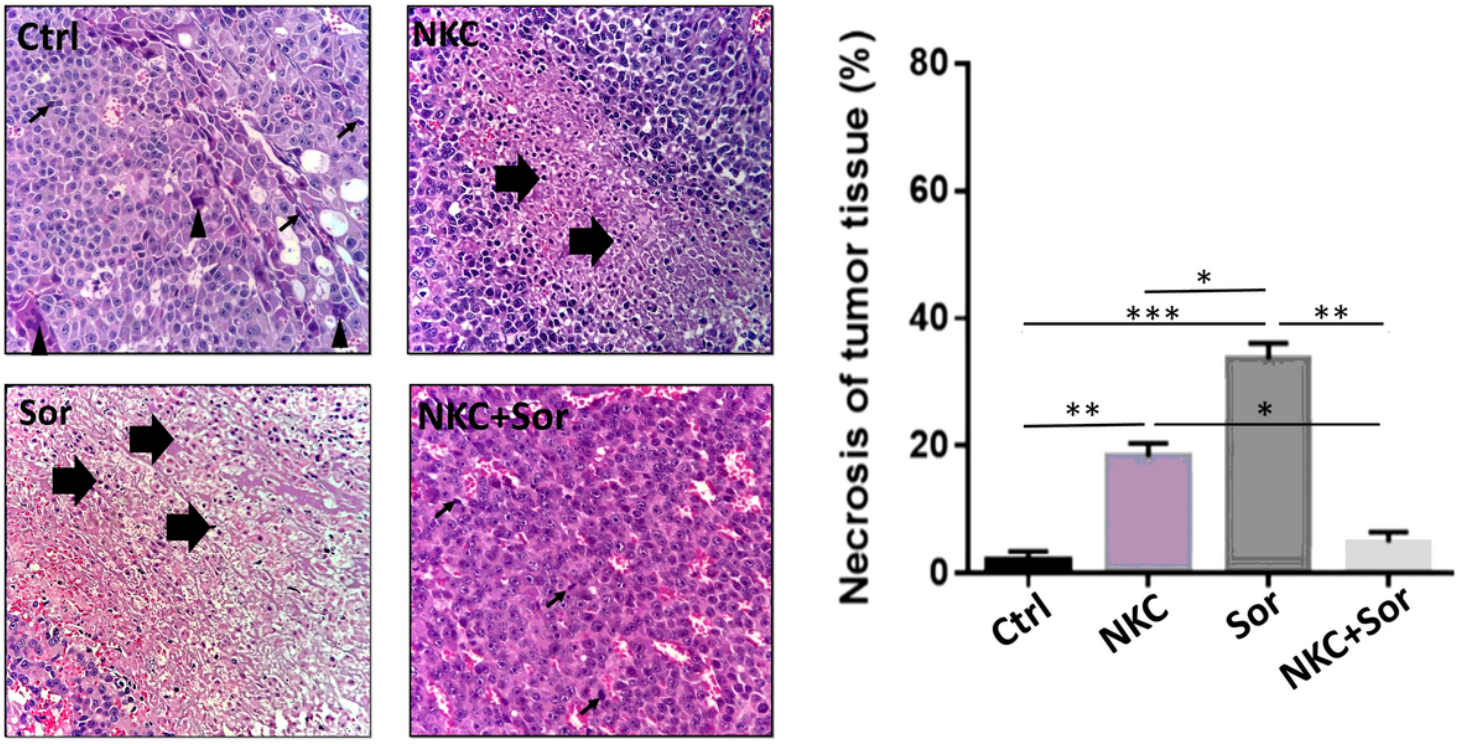

Figure 3

(A) Histopathology of hepatocellular carcinoma xenografts in different treated groups, Ctrl: control, Sor: sorafenib, NKC: natural killer cell. Arrow heads: paelomorphic tumor bizarre cells. Thin arrows: mitotic figures, thick arrows: apoptotic cells, asteroids: necrotic areas. (B) Evaluation of xenograft HCC tumor necrosis by different treatment over 28 days. Statistical analysis shows a significant induction of tumor tissue necrosis in the Sor or NKC treated group, but combination of NKC and Sor had no considerable incremental effect on the necrosis of tumor tissues compared control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

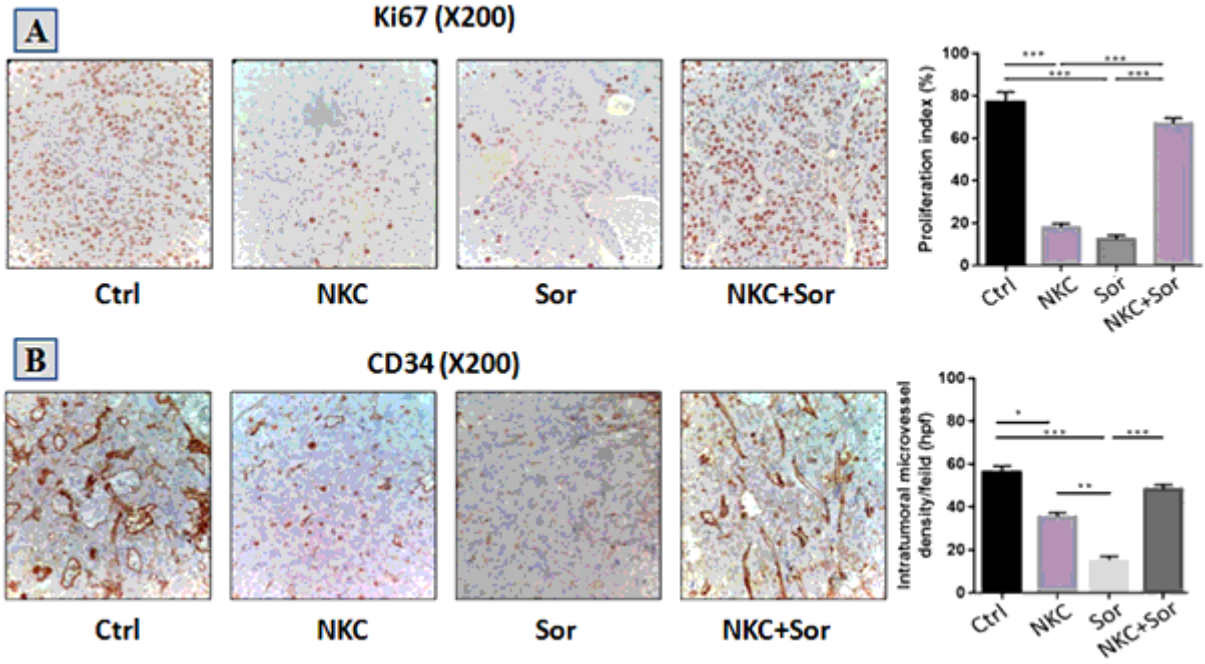

Figure 4

Effects of different treatment on intratumoral angiogenesis and tumor cell proliferation in hepatocellular carcinoma xenografts in nude mice by immunohistochemical (IHC) analysis of the CD34 and Ki67 marker, respectively. The brown color represents positive staining for CD34 and Ki67 cell markers. (A) Sor (30 mg/kg per day) or NKC (5 × 106 cells/mouse, two times a week apart) illustrated significant suppression of tumor cell proliferation compared with control groups, but combination of NKC and Sor showed no synergic effect against tumor proliferation. (B) Statistical analysis showed significant difference between Sor and CGin decreasing of tumor vessels (*** $p < 0.001$), also NKC showed considerable inhibition of tumor angiogenesis (* $p < 0.05$). But combination of NKC and Sor had no meaningful effect on micro vessel density of tumor tissue. Ctrl: control, Sor: sorafenib NKC: natural killer cell * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

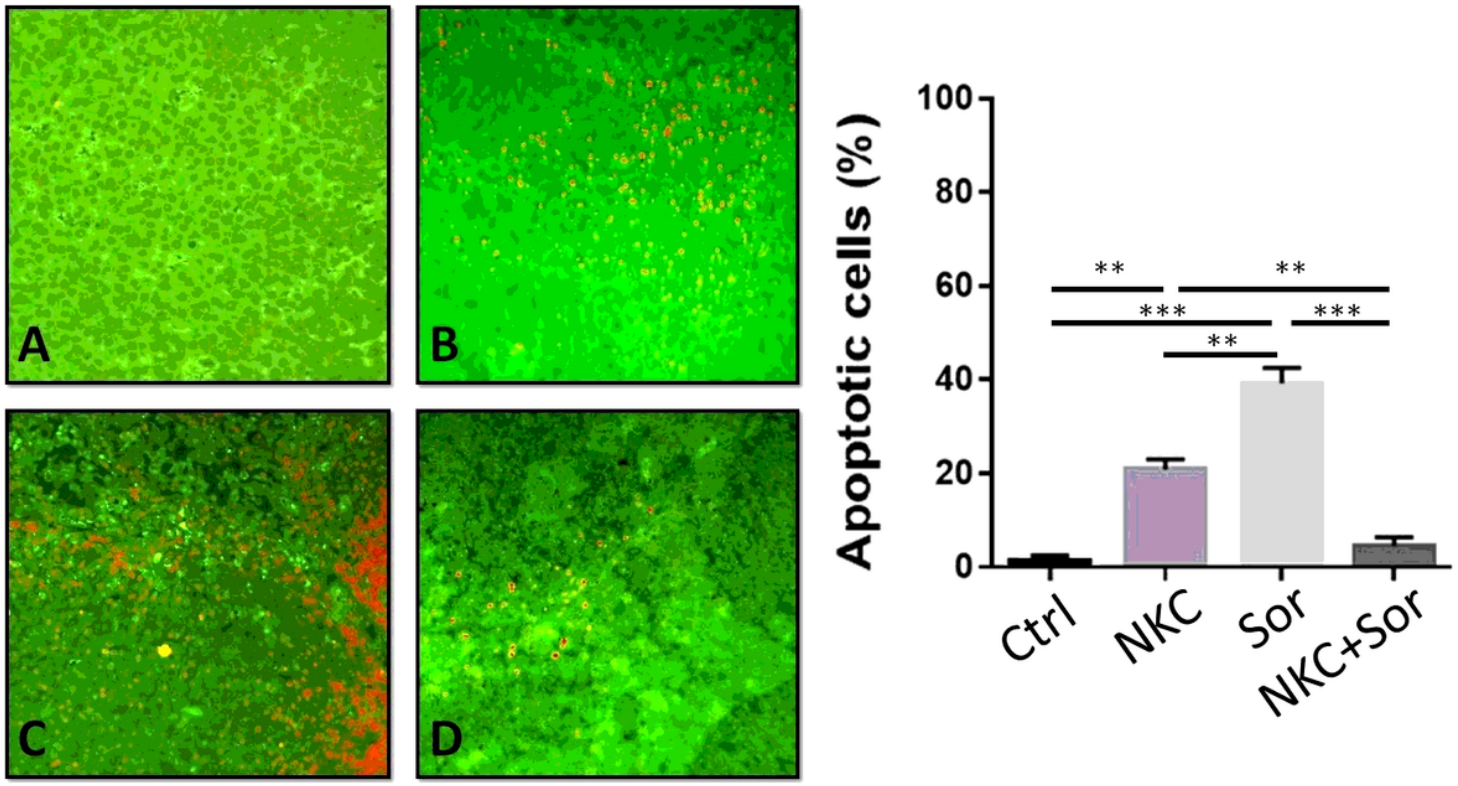

Figure 5

TUNEL staining in HCC xenografts nude mice model (magnification, ×400) A: Ctrl, B: NKC: natural killer cell, C: Sorafenib, D: NKC+Sor. TUNEL assay was utilized to quantify the apoptotic cells in different groups which represents with red florescent-stained nucleus cells. The quantification of the apoptotic cells showed in the diagram that illustrated the proportion of apoptotic-positive cells significantly increased in alone Sor (, *** $p < 0.001$) and NKC (** $p < 0.01$) treated mice compared with those of other experimental groups. Combinational treatment of HCC nude mouse model of HCC by NKC and Sor at specially injected doses had no significant effect against tumor growth by enhancing the apoptosis.

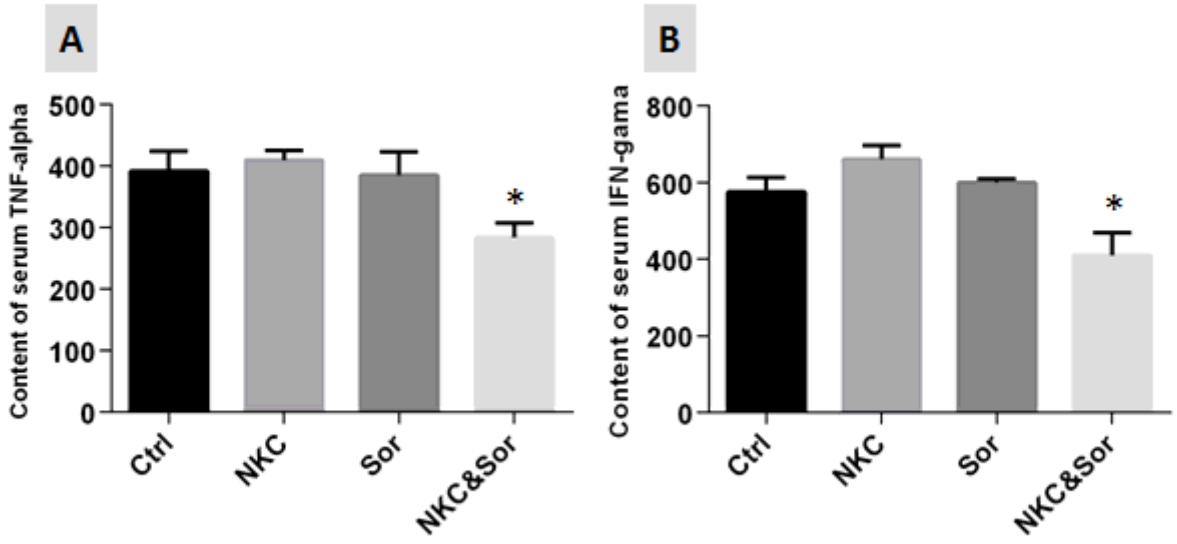

Figure 6

The serum levels of TNF-α (A) and IFN-γ (B) in different treated mouse models of HCC. Ctrl: control, Sor: sorafenib NKC: natural killer cell. The serum levels of TNF-α and IFN-γ in the combined NKC plus Sor treated group was significantly lower than alone NKC or Sor treated group compared Ctrl (* $p < 0.05$).

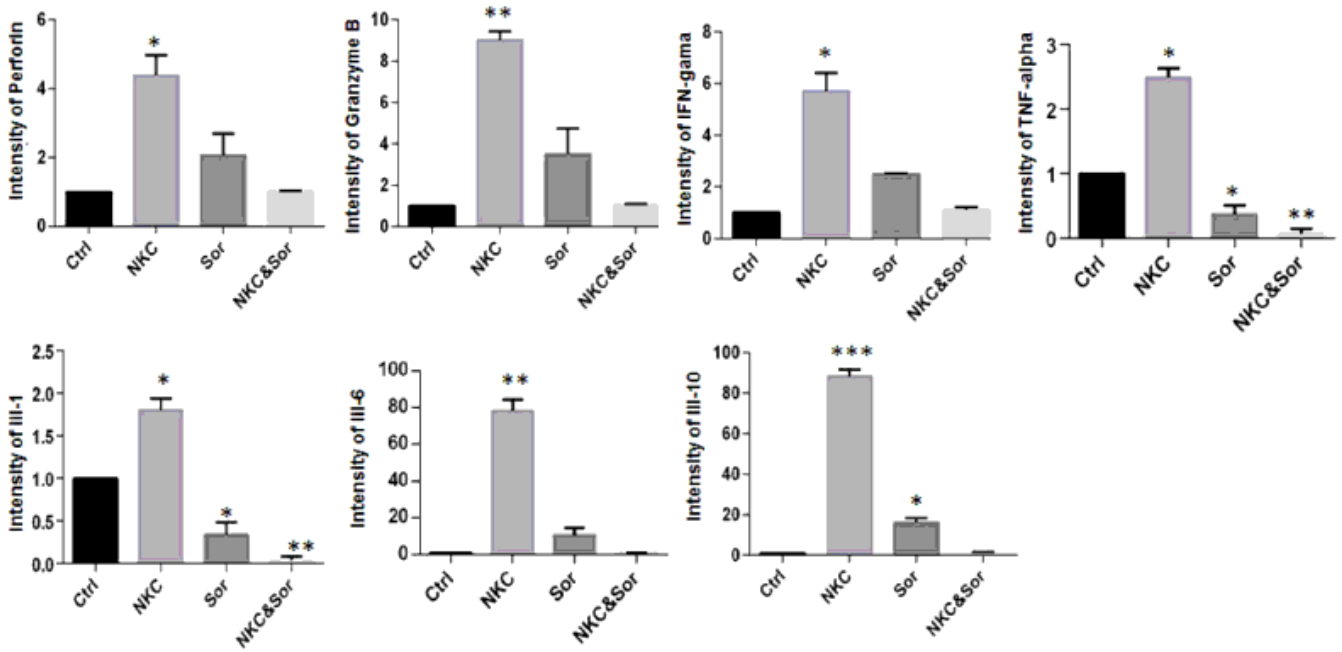

Figure 7

The expression levels of human Perforin, Granzyme B, IFN-γ, TNF-α, IL-1, IL-6, IL-10 genes in xenograft HCC tumor tissues under different treatments; Ctrl: control, Sor: sorafenib, NKC: natural killer cells. NKC significantly increased the expression of all factors and a significant reduction was found in IL-1, IL-10 and TNF-α expressions in the sorafenib treated group as compared with the control group. Combinational NKC and Sor treated group showed the least expression levels of all factors relative to the HCC animal models receiving alone NKC or Sor. Expression of mRNA was quantified by real time PCR and normalized to GAPDH gene.