Research Article

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Differential effects of choline on TLR2/4 mediated signaling through possible regulation of Toll-interacting protein in hepatocellular carcinoma cell lines

https://doi.org/10.1515/tjb-2023-0282
Received December 23, 2023; accepted March 20, 2024;
published online May 30, 2024

Abstract

Objectives: Toll-like receptor (TLR) mediated inflammatory status plays an important role in development and progression of hepatocellular carcinoma (HCC). Toll-interacting protein (TOLLIP) has an inhibitory effect on TLR-mediated inflammatory signalling and expression profile of TOLLIP varies between malignancies including HCC. Cholinergic anti-inflammatory pathway (CAP) is an endogenous mechanism that controls inflammatory status via α7nicotinic acetylcholine receptors (α7nAChR). This study aims to investigate the effect of CAP-acting agent choline on TOLLIP and its related TLR-mediated inflammatory response in HCC cells with distinct differentiation stages.

Methods: The expression patterns of *α7nAChR*, *TLR2/4*, *TOLLIP*, *IL6*, *NFkB* genes were evaluated by RT-PCR and ELISA in the presence of choline, along with the real-time cell proliferation and migration in HEP3B and SNU449 HCC cell lines. The interaction between choline and TOLLIP assessed by using *in-silico* analyses.

Results: Choline downregulated TOLLIP in Hep3B and SNU449 cells. However, the expressions of *α7nAChR*, *NF-κB*, *IL-6*, *TLR2* and *TLR4* showed a decreased pattern in well differentiated HEP3B cells, while an increased pattern in poorly differentiated SNU449 cells.

Conclusions: Choline might exert differential effects in TLR2/4-dependent signalling based on the differentiation stages of the HCC cells, suggesting its potential therapeutic effects in earlier stages of HCC which might be result of its partial modulation of TOLLIP.

Keywords: choline; hepatocellular carcinoma; inflammation; toll-like receptor; Toll-interacting protein

Introduction

Liver cancer is one of the common cancers in humans that have many subtypes. Among them, hepatocellular carcinoma (HCC) is the most recurrent malignant liver tumor [1]. Several studies showed that inflammatory status plays an important role in development and progression of the liver diseases including HCC [2]. Environmental factors, either of viral or non-viral origin, are known to have impact on chronic inflammation. HBV or HCV infection, alcohol consumption, high-fat diet, metabolic diseases such as diabetes, are all risk factors for HCC through chronic inflammatory status [3]. During viral infection, chronic inflammation can lead to tissue damage since cytotoxic T-lymphocytes accumulate through platelets at the inflammation site, which are shown to be important mediators of liver damage via hepatocyte proliferation and DNA alterations. The tumor microenvironment can be modified with the accumulation of cytotoxic T-lymphocytes, which may prevent the host immune system response. In addition to this microenvironmental changes, some parts of the HBV genome can be integrated into the host genome and lead to genomic instability, an important enabling characteristic for cancer development [3].

At the molecular level, NF-kB, IL6, STAT3, JNK and TLRs are well known to influence chronic inflammation and HCC development [4]. Toll-like receptors (TLR) are involved in normal immune function of the liver as well as liver pathologies including HCC [5, 6]. Previous data showed that TLR2 and TLR4 expression is increased in liver damage under proinflammatory conditions [5]. Higher expression

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profile of NF-κB and pro-inflammatory mediator signaling pathways are demonstrated in poorly differentiated cells like SNU449 that represents later stages of HCC [7]. Toll-interacting protein (TOLLIP) has an inhibitory effect on TLR2 and TLR4 mediated signalling while regulating the inflammatory pathway [8]. The expression profile of TOLLIP varies between different cancer types including HCC, which may have effect on different TLR mediated signalling process in different cancer cells.

Cholinergic anti-inflammatory pathway is an endogenous mechanism that controls inflammatory status via parasympathetic innervation and activation of nicotinic receptors expressed on inflammatory cells, particularly α7 nicotinic acetylcholine receptor (α7nAChR) subtype [9]. Studies showed that α7nAChR agonists like acetylcholine, choline or nicotine, downregulate intracellular signalling cascades and decrease pro-inflammatory cytokine levels in many inflammatory conditions [10] Parasympathetic innervation of liver and immune cells found within, has essential role in immunological responses via cholinergic anti-inflammatory pathway [11]. Choline participates in synthesis of acetylcholine, which is the main neurotransmitter of cholinergic system, and directly interacts with α7nAChRs at higher doses. Additionally, choline plays an essential role in the phospholipid synthesis. Choline administration causes an increase in choline levels in brain and periphery, and results in prevention of inflammation-mediated organ damage including liver [12]. α7nAChRs are extensively expressed in liver and liver cancer cells and these receptors potentially might have essential roles in regulating physiologic and pathological functions [13].

Although involvement of inflammatory processes in development and progression of liver cancer is well established, studies showed some controversial findings about the role of cholinergic stimulation by choline in liver and carcinogenesis. Nicotine mediated activation of α7nAChR shown to be involved in carcinogenesis along with cell proliferation and *in-vivo* progression of metastasis both in liver and colon cancer [14, 15]. On the other hand, α7nAChR activation has been shown to exert anti-inflammatory effects on development of different pathogenesis and carcinogenesis in liver [16, 17]. Meta analyses showed that choline might exert protective effect against liver cancer development [18]. In contrast, higher serum choline levels are positively correlated with HCC patients compared to control group [19], whereas choline deficiency has been considered as a factor for cancer development via exacerbation of inflammatory status along with oxidative damage [20]. Increased circulating choline and its enhanced uptake were observed in inflammatory diseases and cancer [21]. Besides, toll-like receptor (TLR) activation enhances choline uptake by immune cells through triggering the choline transporter and inhibition of choline transporter reduces cytokine production [22]. It has been stated that balance between anti-inflammatory effects and carcinogenic effects might be crucial for new therapeutic strategies for the treatment of liver cancer [23]. Taken together although α7nAChRs might induce progression of malign cells in liver tissue, modulation of inflammatory parameters via cholinergic anti-inflammatory pathway in pathogenic environment might have beneficial effects on carcinogenesis.

Due to contradictory results of the role of choline in liver cancer studies, choline is thought to function via different pathways in different cellular backgrounds of liver cancer. Therefore, we hypothesized that, choline may have differential effects on the Toll-like receptor mediated inflammatory response in well- and poorly-differentiated cells of hepatocellular carcinoma. Due to the regulatory role of TOLLIP on Toll-like receptor mediated inflammatory signalling, we also assessed the effect of choline on TOLLIP expression. Our results revealed a distinct differential inflammatory response on the evaluated genes in well- and poorly-differentiated HCC cells upon choline treatment, while decreased expression of TOLLIP on both cell types. We propose that choline might have potential therapeutic effects in earlier stages of HCC and although needs further investigation, with our *in-silico* analysis on tollip-choline interaction, we propose that choline might partially show its effects through its possible interaction with TOLLIP protein.

Materials and methods

Cell culture

SNU449 (representing poorly differentiated HCC cells) and HEP3B (representing well differentiated HCC cells) hepatocellular carcinoma cell lines were maintained in appropriate cell medium (RPMI 1640, Sigma Aldrich, R8758 and MEM, Sigma Aldrich M4655) supplemented with heat inactivated FBS (10 %) and penicillin (100 U/mL), streptomycin (100 µg/mL, Gibco, Carlsbad, CA) at 37 °C in 5 % CO_2 incubator.

Cells were seeded in T-75 flasks 24 h before the addition of chemicals incubated for 24 h in complete media. Before the experiments, culture media was replaced and cells were treated with choline chloride (100 µM; Sigma-Aldrich C7017) for RT-PCR and protein analyses.

Proliferation and migration assays

Real-time cell analysis system (xCELLigence RTCA-DP, Acea Biosciences, San Diego, CA) was employed to monitor real-time cell proliferation and migration.

E-plate 16 was used for proliferation assay which coated with sensitive microelectrodes at the bottom of the plate that is suitable for this system. Before seeding the cells, background impedance of 100 µL medium was measured. After that, cells were seeded in E-plate 16 (10,000 cells/well) and incubated in laminar flow cabinet for 30 min. After incubation cells were proliferated for 24 h, then choline treatment was administered at various concentrations (50, 75, 100 and 150 µM). The change in cell proliferation was monitored for 24 h with 15 min intervals.

CIM-plate 16 was used for migration assay which coated with sensitive microelectrodes at the bottom of the plate that is suitable for this system. CIM-plate 16 composed of two chambers that allow monitoring cell migration through the inner membrane from upper chamber filled with serum-free media to lower chamber filled with serum-containing media. Cell media was replaced with serum free media 24 h before the experiments, then, cells were seeded in upper chambers (30,000/well) and incubated in laminar flow cabinet for 30 min. After that choline treatment was administered at various concentrations (50, 75 and 100 µM) and the change in cell migration was monitored for 20 h with 15 min intervals. Negative control groups (cell-free culture medium) were tested in each plate. Real-time changes in electrical impedance are represented as cell index (CI) [24].

TOLLIP protein analysis

SNU449 and HEP3B cells were treated for 24 h with 100 µM choline. TOLLIP protein levels were analysed in cell lysates by Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cusabio, CSB-E14976h) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR) analyses

Total RNA was isolated by an extraction kit (K0732, ThermoScientific) and each sample was converted to complementary DNA using a cDNA synthesis kit (K1622, ThermoScientific). The cDNA samples were amplified with RT-PCR (Biorad CFXconnect) by using forward and reverse primers of *TOLLIP*, *α7nAChR*, *TLR2*, *TLR4*, *NF-κB* and *IL-6* genes (Table 1) [25]. Cycling conditions were 95 °C for 10 min for polymerase activation/denaturation and 40 cycles (95 °C for 15 s, and 50 °C for 60 s for amplification followed by a dissociation stage (65 °C for 5 s then 5 s each at 0.5 °C for increments between 65 °C and 95 °C). House-keeping gene *GAPDH* was used as an internal control for normalization. 2–ΔΔCt method was employed for the relative quantification of mRNA expression [26].

Molecular docking analysis

Molecular docking between TOLLIP protein and the ligand choline was analyzed with SwissDock (http://www.swissdock.ch/docking, accessed on 23 December 2022) [27] with accurate docking type and flexibility for side chains within 5 Å of any ligand atom in its reference binding mode. The protein structure for TOLLIP protein was obtained from Alphadock (https://alphafold.ebi.ac.uk/entry/Q9H0E2, accessed on 15 December 2022) [28, 29] and the structure for choline was obtained from ZINC database (https://zinc.docking.org/substances/ZINC000003079337/, accessed on 15 December 2022). Molecular docking results were analyzed in UCSF Chimera program (https://www.cgl.ucsf.edu/chimera/) for the most probable docking model (Full fitness –1,433.77 kcal/mol, estimated DG -7.91 kcal/mol).

Molecular dynamics simulation

Molecular dynamic analysis was performed by the "protein with ligand simulation" online tool of WebGRO for

Table 1: Primer sequences used in real-time quantitative PCR (RT-qPCR).

Target gene	Forward sequence	Reverse sequence
α7nAChR	5′-CGCCACATTCCACACTAACG -3′	5′-AGACCAGGACCCAAACTTCAG -3′
TLR2	5′-AACTTACTGGGAAATCCTTAC-3′	5′-AAAAATCTCCAGCAGTAAAAT-3′
TLR4	5′-ATTTATCCAGGTGTGAAATCCAG-3′	5′-AATATTAAGGTAGAGAGGTGGCTTAGG-3′
IL-6	5′-ACTCACCTCTTCAGAACGAATTG-3′	5′-CCATCTTTGGAAGGTTCAGGTTG-3′
NF-kB	5′-GACTACGACCTGAATGCTGTG-3′	5′- GTCAAAGATGGGATGAGGAAGG-3′
TOLLIP	5′-ATGGACGACCGCATTGC-3′	5′-ACTTGTCCTCCACCTGCCC-3′
GAPDH	5′- ACCCACTCCTCCACCTTTGAC-3′	5′- CATACCAGGAAATGAGCTTGACAA-3′

macromolecular simulations, which uses GROMACS simulation package [30] (https://simlab.uams.edu/ProteinWithLigand/index.html, accessed on 19 December 2022). For the simulations, AlphaFold structure of TOLLIP (https://alphafold.ebi.ac.uk/entry/Q9H0E2) and the choline topology, which was generated by using ProDRG (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg, accessed on 17 December 2022) were used [31]. GROMOS96 43a1 forcefield was used for the preparation of protein-ligand complex. All the parameters were kept as default (Water model: SPC, Box type: Triclinic, Salt type: NaCl, Neutralization by addition of 0.15 M salt, integrator: Steepest descent, constant temperature: 300 K, pressure: 1 bar, approximate number of frame per simulation: 1,000) except the step number for energy minimization was used as 50,000 and simulation time as 50 ns.

Statistical analysis

One-way analysis of variance analysis (ANOVA) with post-hoc Tukey-Kramer multiple comparison tests (GraphPad Prism 5, La Jolla, CA) were used to compare means of proliferation and migration assays. Student's t-test were used for comparison of two groups for RT-PCR analyses. Data were expressed as mean±S.E.M. and $p<0.05$ was accepted as statistically significant.

Results

Inhibitory effects choline on cell proliferation and migration

Our data showed that proliferation rate of HEP3B cells significantly decreased in choline-treated groups at concentrations higher than 75 μM at 24th hour significantly (Figure 1; $p<0.01$ and $p<0.001$), while the decrease was not significant for SNU449 cells. Migration rate of SNU449 and HEP3B cells decreased in choline-treated groups (Figure 2; $p<0.05$).

Downregulation of TOLLIP expression after choline treatment

TOLLIP mRNA and protein expressions significantly decreased upon 24 h of choline-treatment (100 μM) both in

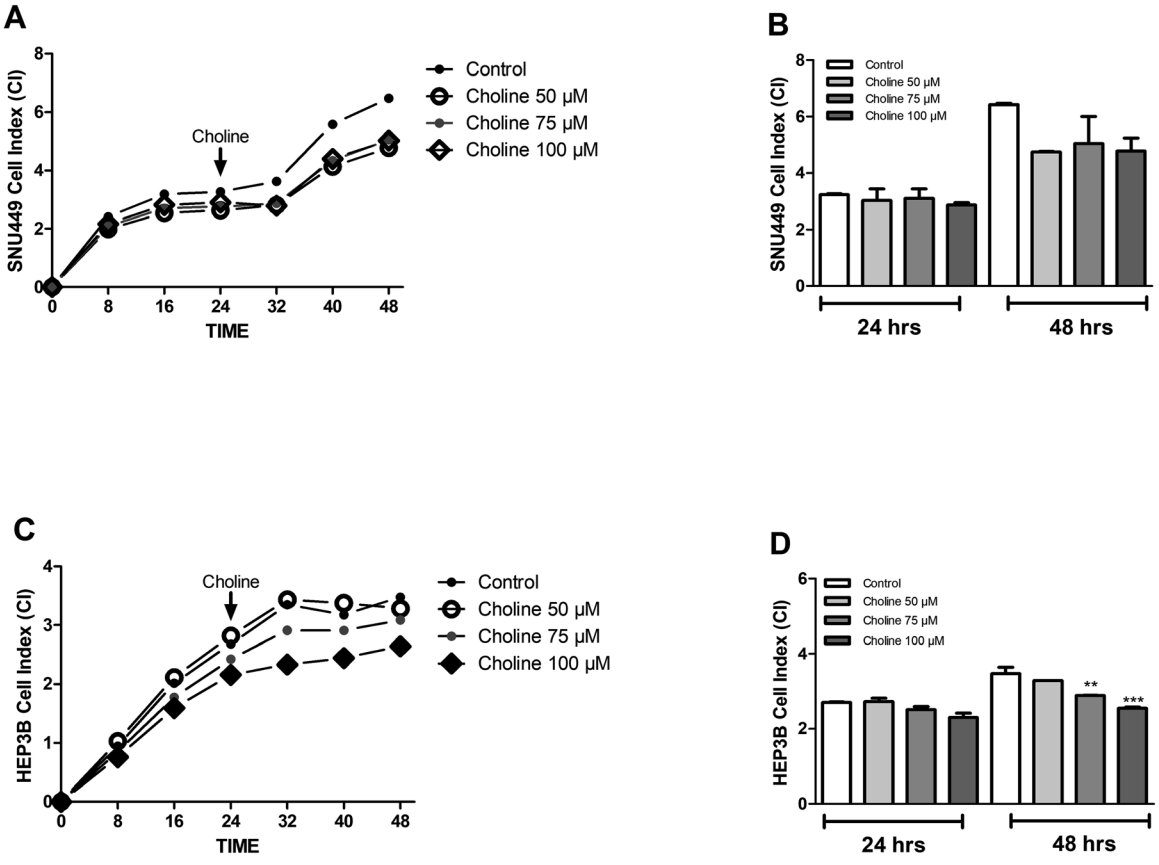

Figure 1: Effects of choline on proliferation rate of SNU449 and HEP3B cells. Shown are line graphs drawn by the averaged data points of the real-time proliferation assay (A and C) and the cumulative data (B and D) of corresponding times after choline administration. Data are shown as mean±S.E.M. (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ vs. control, one-way ANOVA with post-hoc Tukey-Kramer multiple comparison test).

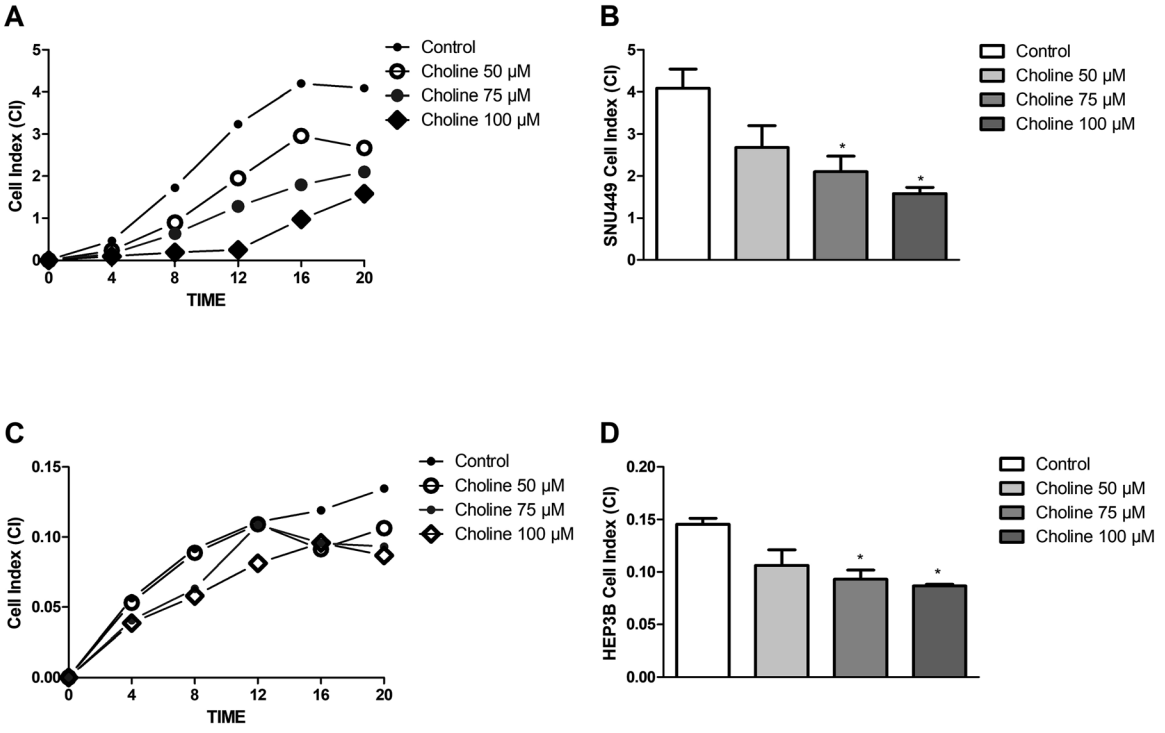

Figure 2: Effects of choline on migration rate of SNU449 and HEP3B cells. Shown are line graphs drawn by from the averaged data points of the real-time migration assay (A and C) and the cumulative data (B and D) of 20th hour after choline treatment. Data are shown as mean±S.E.M. (*, $p<0.05$ vs. control, one-way ANOVA with post-hoc Tukey-Kramer multiple comparison test).

SNU449 and HEP3B cells (Figure 3). Accordingly, basal tollip protein levels were significantly higher in HEP3B control cells compared to that of SNU449 control cells (Figure 3B).

Differential effects of choline on α7nAChR, TLR2, TLR4, NF-κB and IL-6 expressions in SNU449 and HEP3B cells.

α7nAChR, *TLR2*, *TLR4*, *NF-κB* and *IL-6* mRNA expressions were significantly increased upon 24 h of choline treatment (100 µM) in SNU449 cells while significantly decreased in HEP3B cells compared to that of corresponding controls (Figure 4).

In silico protein-ligand simulations for TOLLIP and choline

A possible interaction between tollip protein and choline was evaluated *in-silico*. SwissDock results were analyzed with Chimera and Chimera Model 1.1 was chosen as the most favorable model among others with the estimated values of energy as –115.208 kcal/mol, full fitness as –1,433.77 kcal/mol and ΔG as –7.91 kcal/mol, which together indicates a possibility of interaction (Supplementary Figure 1).

Further molecular dynamic analysis of tollip and choline interaction proposed a similar possible interaction model as obtained from molecular docking analysis (Supplementary Figure 2A). The amino acids glutamine 44, tyrosine 116 and histidine 135 on tollip protein may have the potential to play role in the possible choline interaction. According to the root mean square deviation (RMSD) plot, the protein structure seems to be equilibrated after 20 ns and RMSF plot shows that the fluctuation is minimum between residues 50 and 175 (Supplementary Figure 2B and C).

Although further confirmation is needed, with this study, we propose that choline may partially show its effects through its interaction with tollip protein and further leading to changes in the evaluated inflammatory parameters in hepatocellular carcinoma cells.

Discussion

Inflammatory status, which is known to be involved in development and progression of hepatocellular carcinoma (HCC), differ among HCC cell types. Higher expression of NF-kB, and pro-inflammatory mediator signalling pathways are demonstrated in poorly differentiated cells like SNU449 [7]. Studies showed that choline exerts positive effects on inflammation-mediated organ damage including liver [12].

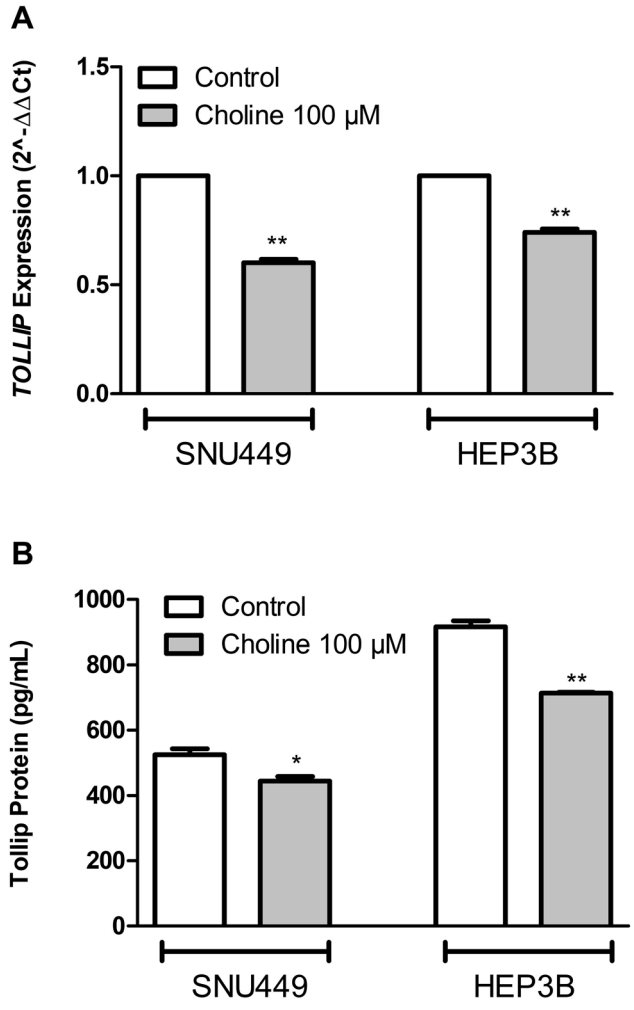

Figure 3: Effects of choline on Tollip expression in SNU449 and HEP3B cells. Shown are the effects of choline (100 µM) on *TOLLIP* mRNA (A) and protein (B) levels in SNU449 and HEP3B cells. $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of mRNA expression. Data are shown as mean±S.E.M. (*, p<0.05; **, p<0.01 vs. control, Student's t-test).

Some studies showed that choline produces a protective effect against liver cancer development [18] and choline deficiency has been considered as a factor for cancer development [20], while in some studies higher serum choline levels are positively correlated in HCC patients [19]. Our data showed that choline treatment exert different effects on HCC cells depending on their characteristics. In poorly differentiated SNU449 cells, representing later-stages of HCC, choline treatment exacerbated inflammatory status by increasing TLR mediated NF-kB and IL-6 pathway at the mRNA level, whereas produced opposite effects in well differentiated HEP3B cells that represent early stages of HCC [7]. These results indicate that choline mediated effects on inflammatory status developed in HCC cells might differ in early and late stages due to their differences in basal expression profile which may explain controversial findings demonstrated with previous studies that investigated involvement of choline in HCC pathogenesis.

Toll-interacting protein (TOLLIP) produces inhibitory effect on toll-like receptors (TLRs) and modulates inflammatory response in liver pathogenesis [8]. TOLLIP has three parts of binding domains, among them coupling of ubiquitin to ER degradation (CUE) domain and TLR interaction demonstrated with structural analysis although underlying mechanisms was not clearly defined [32]. On the one hand, tollip may interact with TLR4 and inhibits TLR-mediated immune response via downregulating NF-κB with CUE domain after high concentrations of LPS-induction. On the other hand, low concentrations of LPS-induction cause TOLLIP translocation to mitochondria in cells and may induce inflammation [33]. As a result, TOLLIP may play distinct roles in modulating inflammation through its different localizations within the cell. Our data indicated that basal tollip protein levels were higher in HEP3B cells compared to SNU449 cells although controversial findings among these cell lines regarding the alteration of TLRs and TOLLIP expressions after choline treatment might partially be explained with localization of tollip within the cell.

Regarding the interaction of TOLLIP with choline there is lack of evidence. Studies showed that increased choline transportation in cells by TLR activation also induces choline-mediated synthesis of phosphatidylcholine (PC) synthesis that involves phospholipid production [22]. Structural analyses suggest that ubiquitin binding via CUE domain of tollip may reduce its phospholipid (preferably phosphoinositide) binding that might be responsible for its translocation to mitochondria [34, 35]. Additionally, higher choline uptake was demonstrated in hepatocellular cell line representing earlier stages of HCC [36]. Our results showed that *TOLLIP* mRNA and protein expressions significantly decreased with choline treatment in both Hep3B and SNU449 cells, however the effect of this decrease totally differs among these cells. These findings might be due to the higher choline uptake in well differentiated HEP3B cells, in which phospholipid synthesis might be increased and somehow affects the location of tollip within the cell leading to differential mRNA synthesis on inflammatory pathway related genes compared to SNU449. In our previous study, we have shown that inhibition of the *TOLLIP* gene intensified the inflammation status in poorly-differentiated SNU449 cells but reduced it in well-differentiated Hep3B cells [25]. The results were similar to the effects of choline treatment on inflammatory status in HCC cells that we show in this study. The molecular docking studies were performed since TOLLIP expression was reduced upon choline treatment and similar effects on

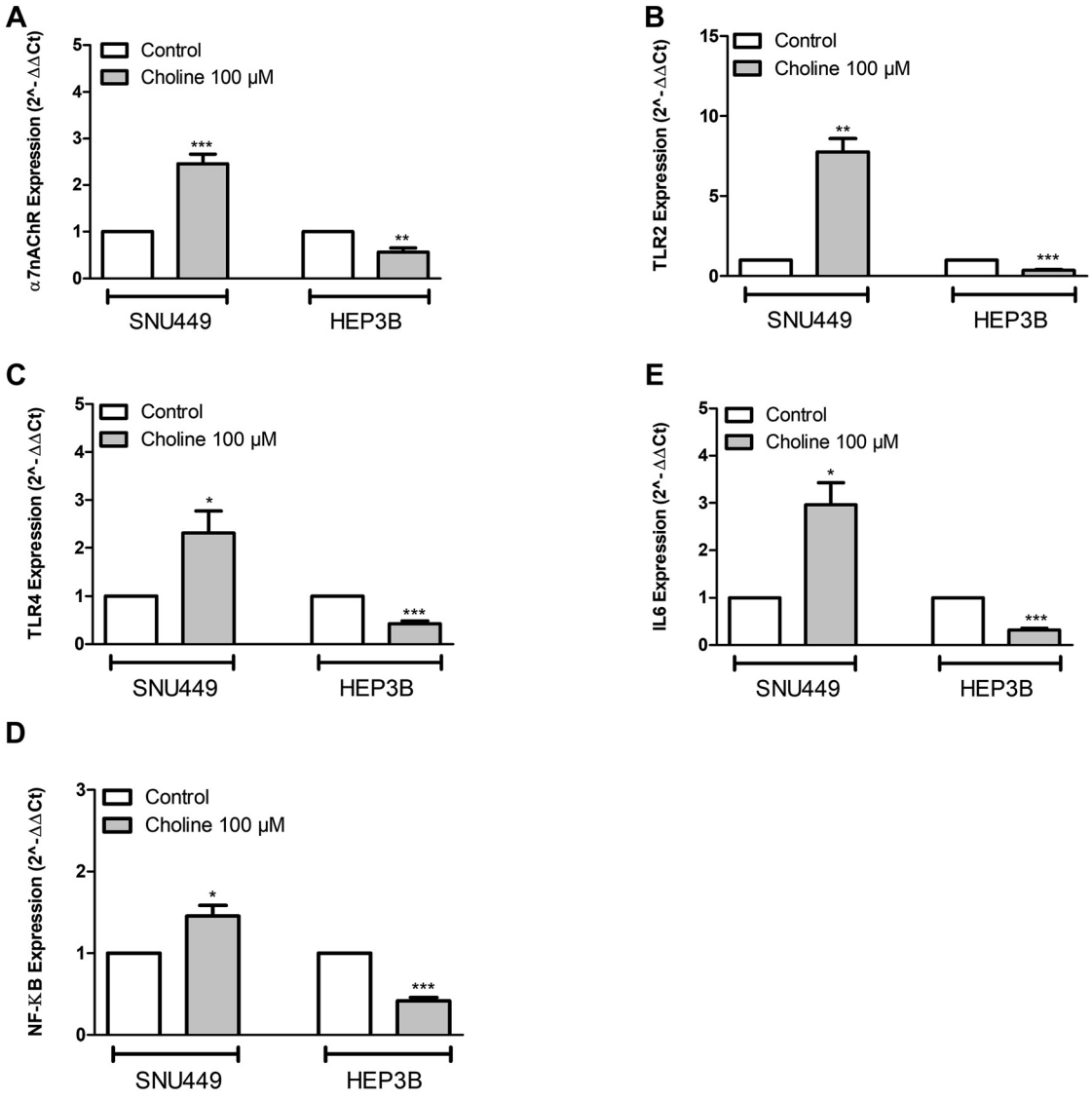

Figure 4: Effects of choline (100 μM) on *α7nAChR, TLR2, TLR4, NF-κB* and *IL-6* expressions in SNU449 and HEP3B cells. Shown are effects of choline on *α7nAChR* (A), *TLR2* (B), *TLR4* (C), *NF-κb* (D) and *IL-6* mRNA (E) levels in SNU449 and HEP3B cells. $2^{\Delta\Delta Ct}$ method was used for the relative quantification of mRNA expression. Data are shown as mean±S.E.M. (*, p<0.05; **, p<0.01 vs. control, Student's *t*-test).

inflammatory status was seen in both choline treatment and TOLLIP silencing, separately. We have also recently shown that the inhibition of TOLLIP rendered SNU449 and Hep3B cells more sensitive to Doxorubicin therapy that is used in TACE therapy [24]. According to our results from *in vitro* and *in-silico* analysis in this study, we propose that choline may partially show its effects on the evaluated inflammatory parameters through its possible interaction with TOLLIP protein and subsequent inhibition of its function, which requires further investigation. A possible interaction between choline and TOLLIP brings the question if choline levels can be important for the effectiveness of chemotherapy regimens in HCC where Doxorubicin is used especially in different HCC subtypes. Either the effect of choline supplementation or the correlation of choline levels with the outcomes of Doxorubicin therapies in different HCC subtypes can be investigated further to reveal if choline can affect the efficacy of Doxorubicin treatments.

Inflammatory processes are known to be involved in development and progression of liver cancer. Nicotine mediated activation of α7nAChR was shown to be involved in carcinogenesis, cell proliferation, metastasis *in-vivo* both in liver and different cancer types [14, 15]. On the contrary, α7nAChR activation has been shown to exert anti-inflammatory effects on development of different pathogenesis and carcinogenesis in liver [16, 17]. According to our

findings, choline treatment upregulated α7nAChR expression while increasing NF-κB and IL-6 expression in SNU449 cell line, although produced opposite effects on HEP3B cells. Choline downregulates α7nAChR expression along with *TLR2* and *TLR4* expressions and ameliorate inflammatory response, which may be through NF-κB pathway, in HEP3B cells. This result indicates that cholinomimetic agents might have potential therapeutic effects in well differentiated – early stages of HCC.

Studies revealed that cells representing later stages of HCC (i.e. SNU-449) having higher proliferative and migratory phenotypes based on their expression of different targets [7]. Acetylcholine esterase (AChE) is an enzyme that breaks down acetylcholine into acetate and choline. Studies showed that inhibition of AChE activity, which might lead to a decrease in choline levels, cause an increase in cell proliferation in HEPG2 and HUH7 cells representing earlier stages of HCC [37] whereas AChE overexpression leads to decreased cell proliferation [38]. Accordingly, our results demonstrate that SNU449 cells presents higher basal proliferation compared to HEP3B cells. Choline treatment significantly decreases cell proliferation compared to non-treated HEP3B cells, although did not produce any significant effect on SNU449 cells. This result might indicate an anti-proliferative effect of choline in earlier stages of HCC. TLR-mediated production of inflammatory cytokines including IL-6 also promotes cell migration [39] via activating STAT3 pathway in liver cancer cells [40]. Our findings showed that SNU449 cells presents higher basal migratory properties compared to HEP3B cells and choline treatment seems to have regulatory effects on cell migration in both cell lines. Controversially, choline treatment significantly increased IL-6 levels in SNU449 cells via NF-κB pathway thus interaction of choline with different intracellular pathways like STAT3 signalling might be involved in the migration process.

Overall, our results show that choline can have different effects on poorly-differentiated and well-differentiated HCC cells and can have potential therapeutic effects in early, well-differentiated HCC. Although we have evaluated tollip both at mRNA and protein levels, we have evaluated the expression of other genes including *TLR2, TLR4, α7nAChR, IL6* and *NFkB* at the RNA level. This constitutes a limitation for our study and further extended analysis at the protein levels are required. Also, analysing the localization of tollip upon choline treatment would be helpful to understand the role of tollip in HCC cells upon choline treatment. However, although more analyses are needed, in line with our hypothesis, showing a totally vice versa pattern at the mRNA levels of several players in TLR signalling pathway in well- and poorly-differentiated cells, might indicate that moderation of Toll-like receptor signalling along with the inflammatory response by choline treatment might differ according to the differentiation state of cells in hepatocellular carcinoma and this effect might be partially exhibited through TOLLIP.

Acknowledgments: The authors would like to acknowledge Prof. Dr. Neşe Atabey for kindly providing the HCC cell lines, Prof. Dr. Metiner Tosun, ELIPS company for kindly providing xCELLigence RTCA-DP equipment and materials and to Fulya Caglar Cirkin for her help in molecular dynamic analysis.

Research ethics: Not applicable.

Informed consent: Not applicable.

Author contributions: EB and ABD designed the project and experimental groups, performed the experiments and collected the data. EB visualized the data. EB and ABD wrote the manuscript.

Competing interests: The authors state no conflict of interest.

Research funding: This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (Grant No: 119Z221 given to ABD).

Data availability: The raw data can be obtained on request from the corresponding author.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/tjb-2023-0282).