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Original Article

Recombinant porcine NK-Lysin inhibits Fascin-1 expression by regulating the MAPK pathway in hepatocellular carcinoma cells

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Abstract

In our previous study, maximum non-toxic concentration (MNTC) of recombinant porcine natural killer lysin (rpNK-Lysin) significantly down-regulated Fascin-1, a prognostic and metastatic biomarker, yet the relevant molecular mechanisms that inhibit Fascin1, was not fully understood. In this study, three different types of hepatocellular carcinoma cell lines (SMMC-7721, MHCC 97H and HepG2) were treated with rpNK-Lysin. Scanning electron microscopy was performed to check its effect on filopodia formation, and the expression of ERK, RSK2, CREB1 and Fascin-1 were determined using qPCR and western blot. Our results showed that MNTC rpNK-lysin successfully down-regulated pERK1/2 which further suppressed the phosphorylation of RSK2 and the transcription factor CREB1, leading to inhibit the CREB transcriptional target Fascin-1, which is involved in filopodia formation. This study confirmed that MNTC rpNK-lysin inhibited metastatic biomarker Fascin1 by down regulating ERK1/2 dependent RSK2 and transcription factor, which further suppressed Fascin1.

Keywords: Fascin1, rpNK-lysin, Metastatic biomarker, Hepatocellular carcinoma, Filopodia

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Introduction

Hepatocellular carcinoma (HCC), the 3rd foremost reason of cancer concomitant deaths, and labeled as 4th most common disease globally (Siegel et al., 2016). High death rates, easy post-operational recurrence, uncontrollability and imperceptible symptoms are the prominent features of HCC along with less than five years' post-operational survival rate (Reynolds et al., 2015). More than 90% cancer mortality is due to metastases rather than tumor at a primer site (Liu et al., 2024). Crucial steps involved in tumor cells metastasis are invasion and migration (Paraiso et al., 2010). Understanding and targeting the molecular and cellular pathways that drive tumor cell invasion and metastasis is therefore of great importance for developing effective therapeutic strategies.

Broad spectrums of mechanism that tightly regulate tumor cells invasion possess a number of cell signaling proteins (Sahai, 2005). The molecular pathogenesis for HCC is though enormously diverse (Wang et al., 2002), many factors trigger and regulate the mitogen-activated protein kinase (MAPK) signaling pathway to transmit signals to the nucleus. Intermediate signaling molecules ERKs, regulate transcriptional factor involved in cell proliferation and cancer development (McCandless et al., 2000). Several extracellular signals stimulate cells by tyrosine and threonine dual phosphorylation and promptly activate serine/threonine kinases i.e. ERK1 (p44mapk) and ERK2 (p42mapk), which are considered to be a crucial part in transmembrane signals transduction which is important for cell division and growth (Shendy and Abell, 2022). MAPK pathway bioactivity is initiated through a signaling cascade phosphorylation. To investigate the role of Fascin1 in HCC metastasis and its relation with MAPK pathway, we studied the expression and phosphorylation level of MAPK signaling cascade molecules.

Ribosomal S6 kinase 2 (RSK2) is activated through phosphorylation by ERK1 and ERK2 (Cho et al., 2009). A Ser/Thr kinase RSK2 confers pro-invasive and pro-metastatic capability to cancer cells, which phosphorylates the several downstream signaling effectors proteins, including the cAMP response element-binding (CREB) protein to control survival and proliferation of the cell (Kang et al., 2010). CREB is a transcription factor having an important role in tumor promotion and progression (Mao et al., 2016). RSK2 regulates CREB through phosphorylation of Ser-133, which facilitates its efficient connection with the basal transcriptional system (Gawecka et al., 2012). In multiple metastatic human tumor cells, the RSK2 - CREB pathway is frequently regulated, causing over-expression of CREB transcription target Fascin1, which is involved in filopodia formation (Li et al., 2013). This crucial connection between MAPK pathway and Fascin1 was investigated in this study.

Fascin1 is a target of CREB1 which binds to actin filaments (Hashimoto et al., 2009), and Fascin1 fasten 10-30 parallel actin filaments together to form a straight, condensed and rigid bundles to form filopodia (60-200 nm in diameter) (Yang et al., 2013). Fascin involvement has been verified in both normal and tumor cells migration of (Sedeh et al., 2010), and has connection with tumor cell invasion (Wang et al., 2002) and metastasis to mature at secondary place (Shibue et al., 2012). Metastasis, aggressiveness, prognosis and survival rate, all are inversely proportional to Fascin expression (Tan et al., 2013). Normal human adult epithelial cells possess low or no Fascin expression while in metastatic cancer its expression is high (Zhang et al., 2022).

Natural killer (NK)-lysin, belongs to the family of saposin-like proteins (Yu et al., 2023), initially isolated from porcine intestinal tissue, is a 9-kD cationic protein secreted by cytotoxic NK cells and T lymphocytes (Zhang et al., 2013), could effectively eliminate a range of cancer cells with no harmful effect on erythrocytes (Yan et al., 2012). Previously, our lab has successfully developed an in-vitro recombinant porcine NK-lysin (rpNK-lysin) eukaryotic expression system and verified its potentiality against HCC cells filopodia (Fan et al., 2016), migration, invasion and metastasis by modulating Fascin1 and Wnt/β-catenin signaling pathway (Fan et al., 2019; Khan et al., 2019). Herein, we determined a possible concealed pro-invasive and pro-metastatic ERK1/2, RSK2 and CREB1 signaling pathway involved in the regulation of Fascin1, and rpNK-lysin successfully down regulated it in-vitro. This study effectively demonstrated that rpNK-lysin is a potential anti-tumor agent which may have potential in the generation of novel anti-neoplastic remedies.

Material and Methods

Preparation of rpNK-lysin

Pichia pastoris system was used to successfully expressed recombinant porcine NK-lysin in our laboratory (Fan et al., 2016), and was subsequently mixed in phosphate buffer saline (PBS), and purified

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using micropore size filter (0.22 μm) and preserved at -20˚C.

Cell line

HepG2, MHCC 97-H and SMMC-7721 human HCC cell lines (Cell bank of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640, DMEM and MEM respectively, supplemented with 10% fetal bovine serum and 1% penicillin / streptomycin and incubated at 5% CO2 and 37˚C temperature.

Scanning electron microscopy

Scanning electron microscopy (JSM-6400, JEOL, Japan) was used to investigate the effect of rpNK-lysin on the ultrastructure of SMMC-7721, MHCC 97-H and HepG2. Placing a sterilized coverslip at the bottom of a six-well microplate earlier, 1×10^5 cells/well were seeded and incubated with the above mentioned conditions for 90% confluency. Post 24 h, the medium was removed and fresh medium with MNTC rpNK-lysin (90.8μg/ml), PBS and medium only (control) were incubated again for 24 h. After discarding the medium, cells were washed thrice with PBS and fixed with 4% glutaraldehyde overnight at 4°C followed by 1% osmic acid for 2 h at room temperature. The samples were dehydrated using graded alcohol and critical point drying. Finally, the samples were sputter-coated with 3 nm of gold and analyzed with SEM.

Real-time quantitative PCR

The total RNA was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. With the help of PrimeScript RT reagent kit and gDNA Eraser and random hexamer primers (TaKaRa Bio, Co, Ltd, Dalian, China), 1μg of purified RNA with an A260/A280 ratio of 1.8–2.0 was reversely transcribed to cDNA. QuantiTect SYBR Green PCR kit (QIAGEN, Germany) was applied to conduct qPCR on IQ-2 type real-time PCR detection system (life, USA) with N gene primers (Table 1). The cycling conditions of PCR were as: 95˚C (5 min), again 95˚C for 10 s (40 cycles), 60˚C (30 s), and 72˚C (6 s). Based on the melting curve analysis, the PCR product was demonstrated to be specific. The target gene expression level was related with that of control group and results were calculated using $2^{-\Delta\Delta Ct}$ method. Each RNA sample was measured two times.

Table-1. PCR Primers used in this study and the PCR product size.

Gene	Primer Sequences (5′—3′)	PCR product size (bp)
Fascin	F: CACAGGCAAATACTGGACGGT R: CCACCTTGTTATAGTCGCAGAAC	101
ERK1	F: ACCTGCGACCTTAAGATTTGTGA R: AGCCACATACTCCGTCAGGAA	90
RSK2	F : GGAGGTGAATTGCTGGATAAAA R: GTATCATCAGGACCATTTGC	383
CREB1	F: ACATTAGCCCAGGTATCTATGCCAG R: CCTGAATGACTCCATGGACTTGAAC	112
β-actin	F : AAATCGTGCGTGACATTAA R: GGAAGGAAGGTTGGAAGAGAGC	180

The reference for Fascin, ERK1, RSK2, CREB1 and β-actin are Liu et al., 2017, Hybel et al., 2020, Zeniou et al., 2002, Zhang et al., 2018 and Fan et al., 2019 respectively.

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Western blotting

After the cells were confluent in 6-well plates, medium was removed and rpNK-lysin at a concentration of 90.8, 45.4 and 22.7 µg/mL were added and incubated for 24 h. Total cell extract was obtained with cell lysis buffer (Beyotime Biotechnology, China). The supernatants were obtained, preserved at -80 °C and protein concentration was determined using bicinchoninic acid protein assay reagent (Beyotime, Shanghai, China). pERK1/2, pRSK2, pCREB1 and Fascin1 (1:1000; Cell Proteintech) and Beta-actin (1:2000; Proteintech) expression levels were checked by Western blotting. Protein bands were conveyed to PVDF membranes (Millipore, Billerica, MA, USA) with 5% BSA in Tris-buffered saline with Tween-20 (TBST) were blocked for 2 h, and with the primary antibodies (Cell Signaling Technology, Inc, USA) incubated at 4˚C overnight. Post washing with TBST (20% Tween-20) thrice, again incubated with secondary antibodies (ComWin Biotech, Beijing, China) at 37°C for 1 h. Finally washing three times with TBST and with the help of enhanced chemiluminescence (ECL), immunoreactive bands were visualized and detected by exposure to Kodak X-ray film (Eastman Kodak, Rochester, NY, U.S.A.). Based on the relative intensities of bands, protein expression levels were compared with the control one.

Statistical analysis

Each experiment was triplicated and the results are shown as mean ± SEM and $P<0.05$ was considered as statistically significant. GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA) was used to analyze the data through one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. To analyze the western blot images gray scale bands, Image J software was used.

Results

Scanning electron microscope analysis of the rpNK-lysin

Scanning electron microscope revealed that the ultrastructure of all the three treated HCC cell lines with MNTC rpNk-lysin (90.8µg/ml) exhibited distinct morphological changes, as shown in Figure 1. Control and PBS treated cells exhibited filopodia scattered on the cell membrane of all the three HCC cell lines with excellent cell to cell contact among neighboring cells (Figure 1, control and PBS). In contrast, the MNTC rpNK-lysin treatment exhibited shrinkage of the entire cell and obvious alteration in cell to cell contact among adjacent cells. Moreover, the treated cells significantly showed rare filopodia formations on cell surface compared with the untreated (control) and PBS treated cells.

Effect of rpNK-lysin on the MAPK pathway and Fascin1 gene expression level

The MAPK pathway genes (ERK1 and 2, RSK2 and CREB1) and their downstream target gene Fascin1 expression level was checked and compared between treatment and control group in different HCC cell lines (SMMC 7721, MHCC 97H and HepG2). The results showed that MNTC rpNK-lysin successfully down-regulated ERK1 and ERK2, its substrate gene RSK2 and its downstream transcriptional factor gene CREB1 which has a direct impact on Fascin1. As shown in Figure 2, the mRNA expression of MAPK genes in SMMC 7721 has successfully decreased by rpNK-lysin treatment in concentration dependent manner which further suppresses the expression of Fascin1, a metastatic bimarker responsible for filopodia formation, invasion and metastasis. The above results established that rpNK-lysin inhibited ERK1 and ERK2 expressions that are responsible for its substrate gene RSK2 regulation, which induces transcriptional factor gene CREB1 and ultimately blocks the expression of Fascin1.

Moreover, the results of other two cell lines, MHCC 97H and HepG2 as shown in Figure 3 and 4, are in-consistent with that of SMMC 7721, which induces the potentiality of rpNK-lysin against HCC cells.

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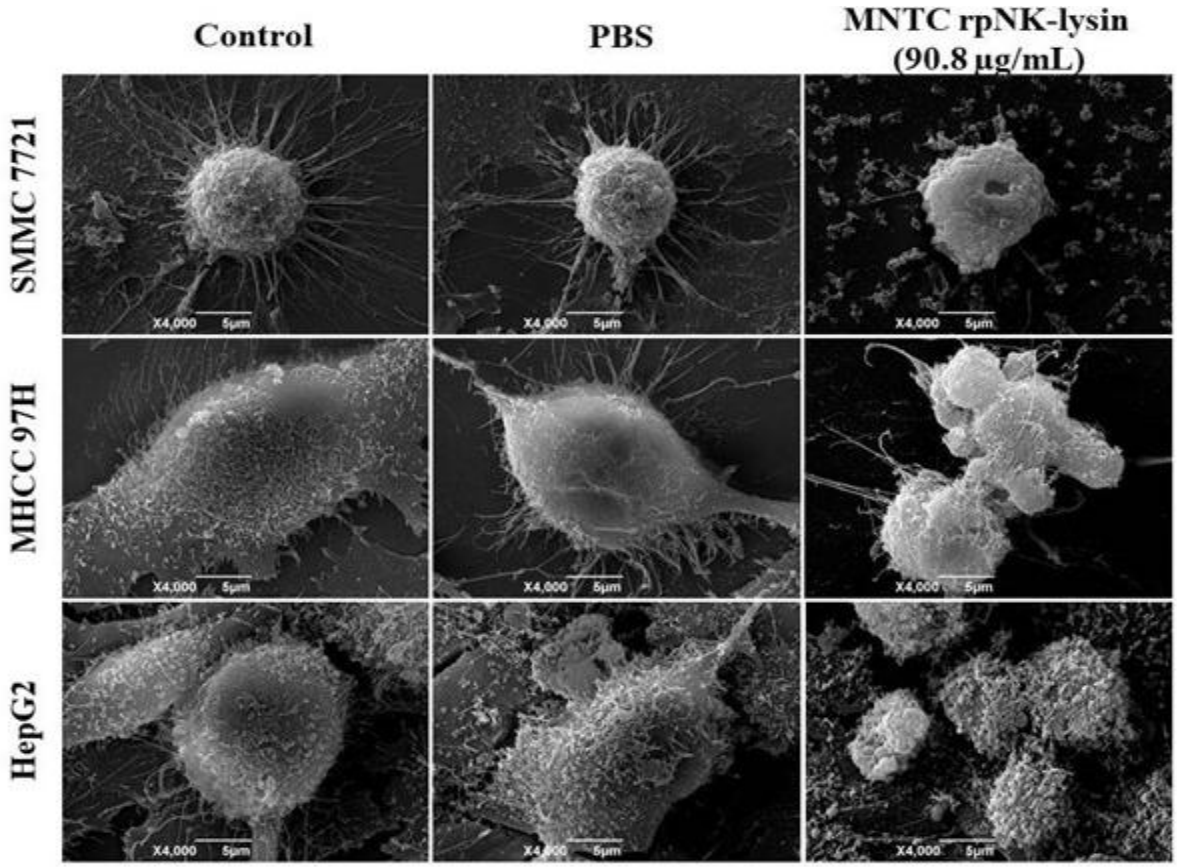

Figure-1. Scanning electron microscopy analysis of three HCC cell lines (SMMC-7721, MHCC-97H and HepG2) incubated with the medium only (control), PBS and MNTC rpNK-lysin (90.8µg/ml) in medium for 24 h. Compared with control and PBS, MNTC rpNK-lysin has shrunk the cell and inhibited the filopodia formation.

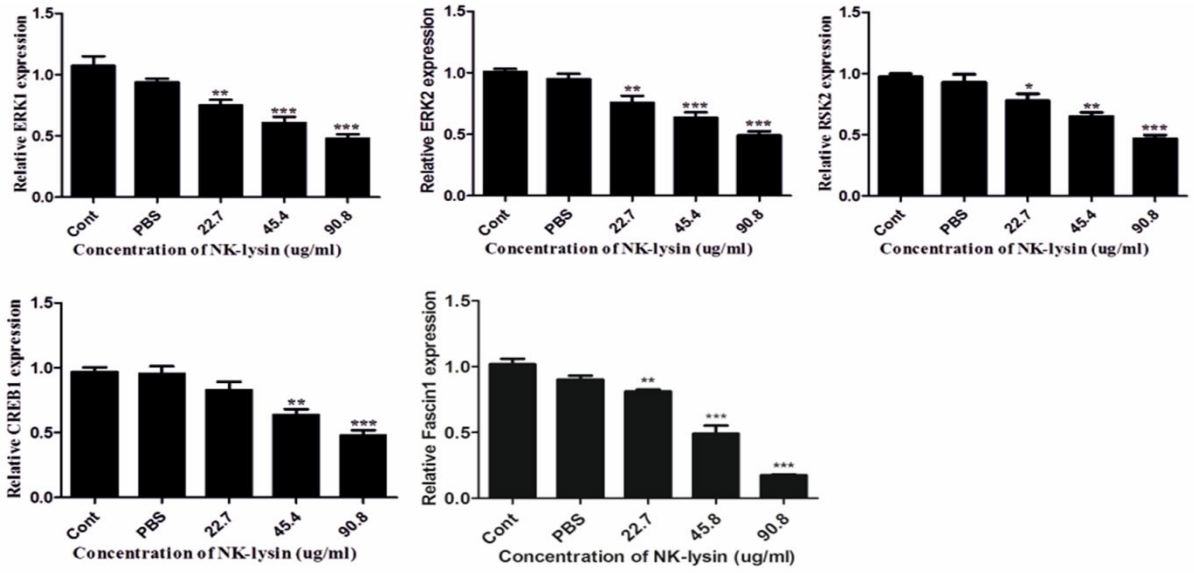

Figure-2. The effect of rpNK-lysin on relative MAPK pathway genes (ERK1, ERK2, SRK2, CREB1) and Fascin1mRNA expressions of SMMC 7721 cells. The expression of all the MAPK pathway in the rpNK-lysin treated groups were significantly decreased in dose dependent manner compared with the control and PBS group (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

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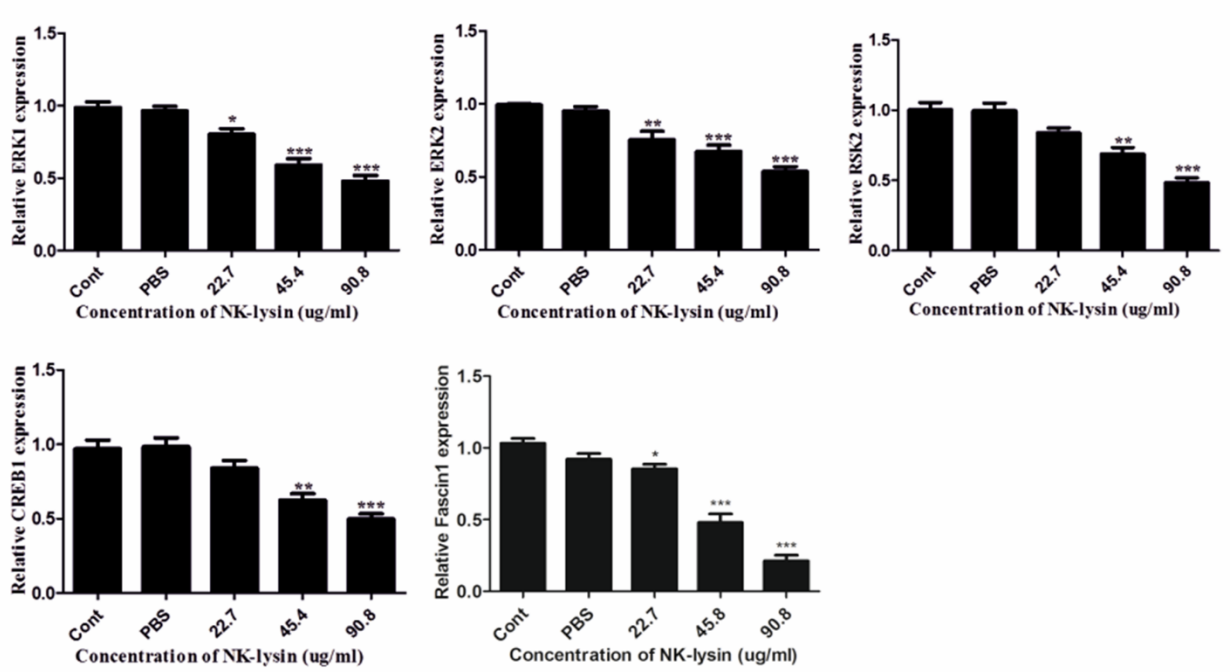

Figure-3. The effect of rpNK-lysin on relative MAPK pathway genes (ERK1, ERK2, SRK2, CREB1) and Fascin1mRNA expressions of MHCC 97H.

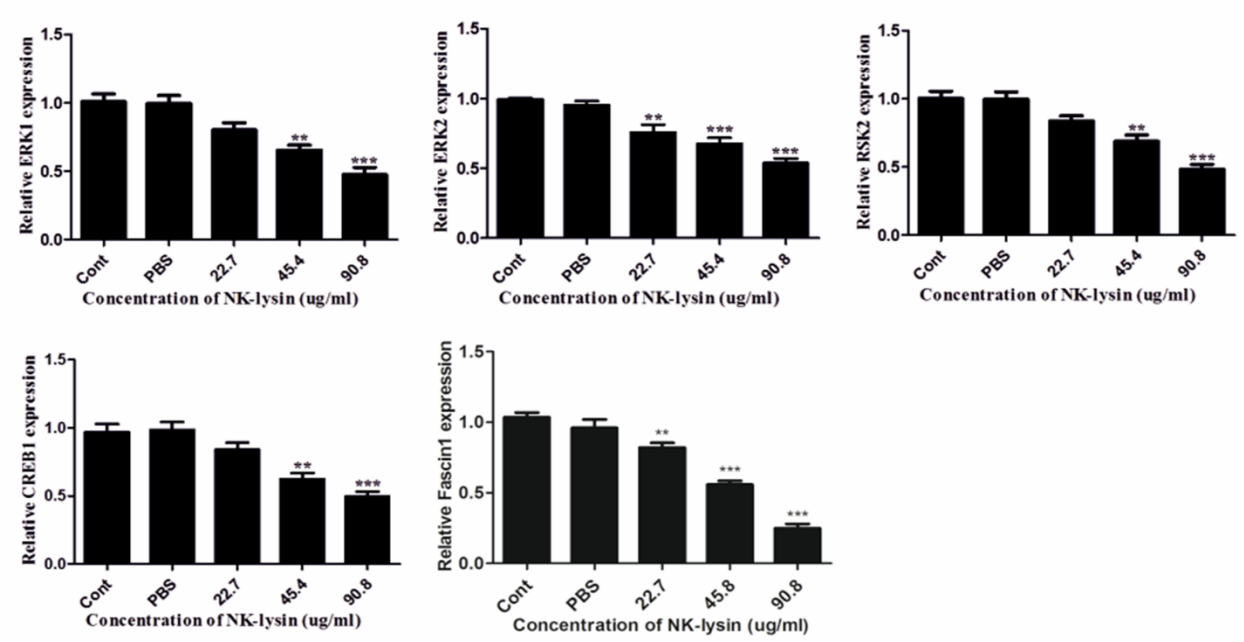

Figure-4. The effect of rpNK-lysin on relative MAPK pathway genes (ERK1, ERK2, SRK2, CREB1) and Fascin1mRNA expressions of HepG2.

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Effect of rpNK-lysin on the protein expression level

The efficacy of rpNK-lysin against MAPK pathway and Fascin1 at protein expression level in three different HCC cell lines was checked through western blotting, which induced the results of PCR. The Phosphorylation of MAPK pathway protein and Fascin1 expression was investigated and compared between control and rpNK-lysin treatment groups. pERK1/2, tERK, RSK2 and CREB1 were down-regulated by rpNK-lysin in SMMC 7721 (Figure 5), MHCC 97H (Figure 6) and HepG2 (Figure 7), which further suppresses Fascin1 expression. Results from all three cell lines indicate that rpNK-lysin exerts its potential effect by suppressing the MAPK pathway, which is involved in the regulation of fascin-1 expression.

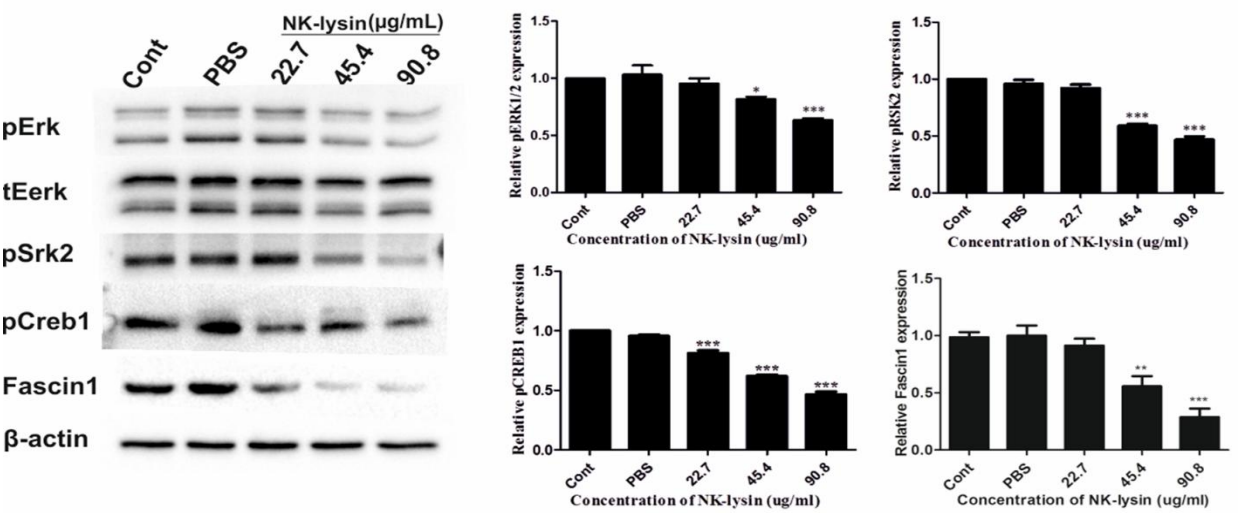

Figure-5. Expression of MAPK pathway protein (pERK1/2, pRSK2 and pCREB1) its relation with Fascin1 and β-actin as a house keeping protein in SMMC 7721 cell line. The expression of all the MAPK pathway in the rpNK-lysin treated groups were significantly decreased in dose dependent manner compared with the control and PBS group (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

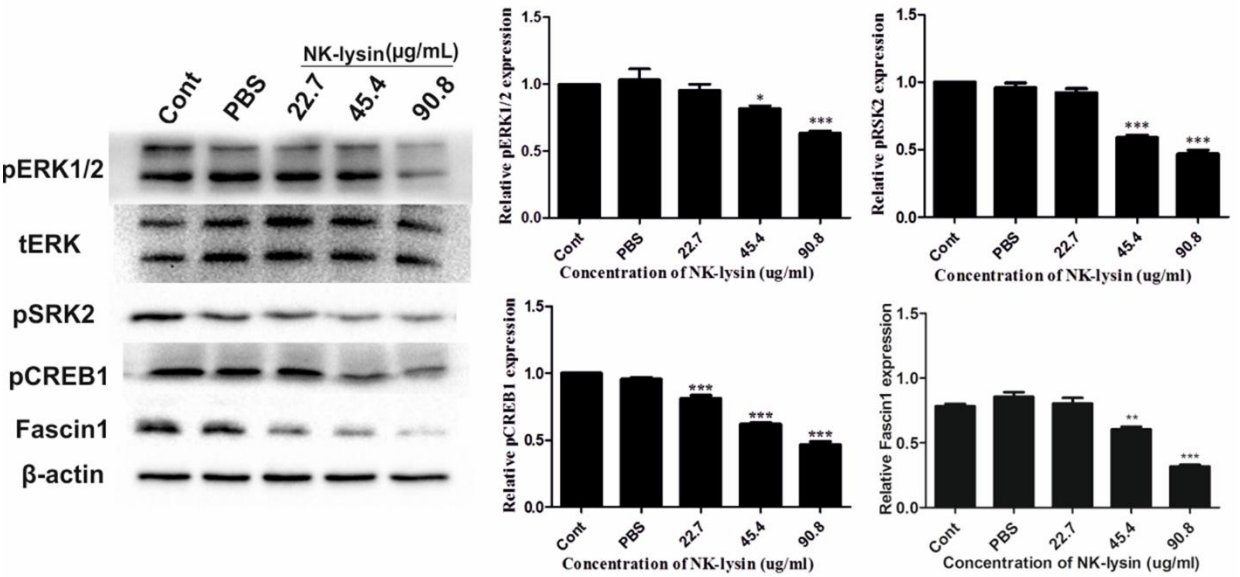

Figure-6. Expression of MAPK pathway protein (pERK1/2, pRSK2 and pCREB1) its relation with Fascin1 and β-actin as a house keeping protein in MHCCC 97H HCC cell line.

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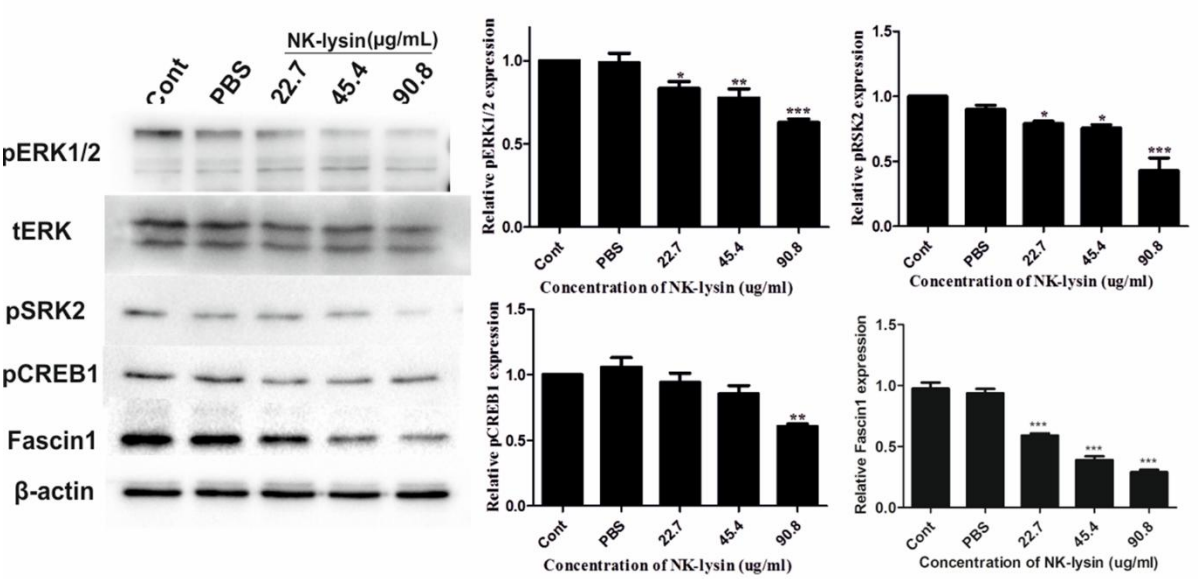

Figure-7. Expression of MAPK pathway protein (pERK1/2, pRSK2 and pCREB1) its relation with Fascin1 and β-actin as a house keeping protein in HepG2 HCC cell line.

Discussion

Patients suffering from HCC have limited survival rate, post operational recurrence and metastasis and the morbidity and mortality due to HCC is alarmingly growing throughout the globe (Reynolds et al., 2015). High recurrence potential and metastasis are general characteristics of liver cancer and foremost resistance in its treatment (Duseja, 2014). Cancer mortality rate is prominently due to metastasis and not due to tumors in the primary site (Chambers et al., 2001), which needs special attention to develop drugs that inhibit invasion and metastasis. Besides substantial and promising research findings, much remained to be explored in combating invasion and metastasis. Consequently, the development of novel drugs for the treatment of liver cancer is urgently needed (Wang et al., 2013).

In humans, natural killer cells and CTLs body stimulated by interleukin 2 (IL-2) secret a cationic antimicrobial peptide called NK-lysin. It has been reported in previous studies that NK-lysin possesses a potent property against bacteria and virus, and it also might have tumor resistance properties as well (Yu et al., 2023). Our study also showed that rpNK-lysin has cytopathic effect on HCC cells and constrains filopodia formation (Fan et al., 2016). Drugs having specificity for the cancer cells with nominal cytotoxicity for healthy cells are extremely momentous for tumor treatment. rpNK-lysin had shown selective cytopathic effect on SMMC-7722, MHCC 97H and HepG2 at maximum non-toxic concentration (MNTC = 90.8 μg/mL), and therefore had a heavy blow on their division (Khan et al., 2019). On molecular level, it was confirmed that as compared to untreated control groups, MNTC rpNK-lysin significantly suppressed adhesion, invasion and migration capacity of HCC cells by suppressing Fascin1 that further blocked Wnt/β-catenin signaling pathway that regulates β-catenin degradation, which reduced MMP-2 and MMP9 expression (Fan et al., 2019; Khan et al., 2019). Herein, in this work we investigated the hidden pathway and the effect of MNTC rpNK-lysin to regulate Fasin1 which further activates Wnt/β-catenin signaling cascade and regulates HCC cells adhesion and metastasis as discussed above.

Abnormal activation of various signaling pathways is engaged in carcinogenesis; MAPK, a well-known pathway commonly related with the induction of tumor metastasis. MAPK cascade activation plays a crucial and cardinal role in various cell division related signaling pathways regulations. In un-stimulated cells; MAPK is in an inactive form, Erk (½) mitogen-activated protein (MAP) kinase pathway performs a vital part to control cell division (Meloche and Pouysségur, 2007). It has been reported that the phospho-activation of various kinases and transcription factors regulates drug resistance and metastasis (Liu et al., 2015). Among them, the

serine/threonine kinases ERK1 (p44mapk) and ERK2 (p42mapk) are promptly activated in cells which are stimulated by different extracellular signals by causing dual phosphorylation of tyrosine and threonine residues, results in integration and transmission of transmembrane signals required for growth and differentiation (Shendy and Abell., 2022). To assess the role of MAPK pathway in the Fascin1 activation, we investigated the phosphorylation level of the MAPK signaling molecules pERK1/2, pRSK2 and pCREB1.

An important signaling node induced and activated by the pERK1 and 2 is RSK2 (Hashimoto et al., 2009), which, further stimulates various down-stream transcription factors including CREB1 (Mao et al., 2016), which binds to Fascin promoter molecule to up regulate its expression (Li et al., 2013). Previous study has been demonstrated that pERK1/2 and pRSK2 are involved in the invasion/metastasis (Mao et al., 2016). Mao et al., 2012 proved that ERK1/2 phosphorylation has been increased in MCF-7 breast cancer cells which induce RSK2 phosphorylation and regulates metastasis, required for poor prognosis while Stratford et al. (2012) confirmed that its over-expression is directly related with limit disease-free survival. Our study showed that rpNK-lysin has successfully decreased the expression of pERK1/2 and its downstream substrate pRSK2 in does dependent manner.

Phosphorylated RSK2 is translocated to the nucleus, where it causes the phosphorylation of numerous nuclear proteins, including CREB (Lee et al., 2025). CREB1, an oncogene located on 2q34 human chromosome, has been recognized as involved in various tumor cellular processes including proliferation, invasion, and metastasis (Sakamoto and Frank, 2009). Abnormal CREB1 expression in multiple tumor types has been associated with the development and progression (Zhang et al., 2005), while suppression of CREB1 prevent liver cancer cell migration (Yang et al., 2013). Our study also showed similarity in which there is high expression of CREB1 in control group and was efficiently down regulated by MNTC rpNK-lysin in all of the three HCC cells.

In different cancer metastatic cells, RSK2-CREB pathway is usually activated which causes up-regulation of a CREB transcription target Fascin1 (Li et al., 2013) Fascin1 tightly packed actin into bundles (Hashimoto et al., 2009). Our previous work demonstrated the role of MNTC rpNK-lysin in the inhibition of Fascin1 which is involved in the invasive and metastasis of HCC cells (Fan et al., 2019; Khan et al., 2019) because Fascin1 over-expression and carcinoma metastases are reversely proportional, as stated by Wang et al. (2017) and Hashimoto et al. (2006). These works elaborate that CREB1 is recognized to induce and activate Fascin1 transcription in carcinoma cells, as described by Kim et al. (2010) and Hashimoto et al. (2009) proposing that pERK1/2 phosphorylates RSK2 which in turn phosphorylates CREB1 to increase Fascin-1 expression, which is involved in filopodia formation, cell migration, invasion, and metastasis. Although our findings demonstrate a strong association between rpNK-lysin treatment and inhibition of the pERK1/2 → pRSK2 → pCREB1 → Fascin-1 pathway, this study is limited by its inability to establish a direct causal relationship, and elucidating the exact mechanism highlighting the need for further mechanistic investigations.

Conclusion

ERK1/2, RSK2-CREB-Fascin-1 pathway is a favorable therapeutic target in metastatic cancers. This study demonstrated that Fascin1is activated by MAPK pathway in which ERK1/2 starts a series of phosphorylation cascade of RSK2 and its downstream transcription factor CREB1, which ultimately activate Fascin 1, resulted in filopodia formation. Fascin1 activate β-catenin which regulates metastatic genes MMP2 and MMP9, responsible for ECM degradation to provide a path for filopodia and HCC invasion and metastasis. Furthermore, MNTC rpNK-lysin inhibited the expression of all these genes which results in the suppression of filopodia formation and ECM degradation needed for invasion and metastasis of HCC cells. This is an important finding, suggesting that rpNK-lysin could be an important substitute for HCC treatment, and highlighting the necessity of advancing its development as anti-cancer therapy.

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Conflict of Interest: None.

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Contribution of Authors

Khan A, Fan K & Li H: Conceived idea and designed the experiment.

Khan A: Performed the experiments, collected & analyzed the data and drafted the manuscript.

Na S, Sun P, Maqbool B, Khan A, Ali Q & Ullah R: Literature review, analyzed data and manuscript editing.

All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final draft.

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