RESEARCH ARTICLE

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The Effect of LL37 Antimicrobial Peptide on *FOXE1* and *lncRNA PTCSC 2* Genes Expression in Colorectal Cancer (CRC) and Normal Cells

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Abstract

Objective: As an innate immune system component, antimicrobial peptides (AMPs) exert various effects, such as anticancer properties. This study aimed to evaluate the LL37 AMP anticancer effect against colorectal cancer (CRC) cells and the expression of *FOXE1* and *lncRNA PTCSC2* genes. **Methods:** The LL37 AMP was purchased from GenScript USA, Inc. Various CRC cell lines (HCT-116, HT29, WiDr, and SW742) were cultured in the DMEM medium. Various concentrations ranging from (5-400) µg/mL of LL37 AMP were prepared, added to cell cultures, and incubated for (24 and 48) hours. A nontoxic level of 30 µg/mL of LL37 was investigated for *FOXE1* and *lncRNA PTCSC2* gene expression. **Results:** At 24 hours, the (50 and 90) % lethal concentrations of LC50 and LC90, respectively, of LL37 against NCM460 normal cells were (640 and > 640) g/mL. Additionally, these values at 48 hours included (160 and > 640) µg/mL, respectively. After 24 hours of treatment, the LC50 and LC90 of LL37 AMP against CRC cell lines included (20 and 200) µg/mL. The LC50 and LC90 of the LL37 at 48 hours included (20 and 50) µg/mL and at 72 hours. they included (~10 and 40) µg/mL, respectively. *FOXE1* but not the PTCSC 2 gene expression was significantly higher in CRC cells than normal cells (NCM460 and HaCaT). The LL37 AMP significantly decreased *FOXE1* gene expression by 1.95-fold in CRC cells ($p < 0.001$). **Conclusion:** The *FOXE1* gene can be considered a biomarker of CRC development. The expression of *FOXE1* but not the PTCSC2 gene was significantly affected by the LL37 AMP. The effects of LL37 AMP against CRC cells were time and dose-dependent. Future studies are warranted to verify these effects.

Keywords: Colorectal cancer- antimicrobial peptides- LL37- anticancer traits

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Introduction

Colorectal cancer (CRC) occurs in two forms: sporadic 80 % and inherent 20 %, accounting for nearly 10 % of cancer deaths worldwide (D'asheesh et al., 2021; Graham et al., 2017). CRC is more likely to show up and get worse if you are overweight, eat much salt and not enough vegetables, get older, smoke, and don't move around enough. Unfortunately, these behavioral changes have lowered the age of CRC to younger generations (Chen et al., 2018; Wu et al., 2021). Therefore, genetic and epigenetic factors play a role in CRC's development. The alterations in the expression of various genes evoke epithelial inflammation and cell division. Non-coding RNAs (IncRNAs) include > 200 nucleotide sequences that inhibit gene expression via adenylation signals and regulatory transcription factors.

The effect of IncRNAs is exerted in specific regions and periods (Ding et al., 2014; Zhu et al., 2016; Statello et al., 2021). Furthermore, these sequences interact with DNA, RNA, and proteins and control various cellular functions. Considering the vital role of these biomolecules in normal cellular activities, the disturbance in their position and regulatory roles will lead to cell division and progress to malignancy and cancer (Carlevaro-Fita et al., 2020; Wang et al., 2017). Hence, their expression profiles are possibly different between normal and cancerous cells. Nonetheless, in some instances, the IncRNAs also cause the inhibition of cancer regulatory genes, thereby predisposing them to progression into tumor conditions. Moreover, the role of mutated IncRNAs in cancer development is unclear.

The *FOXE1* gene is the thyroid-2 transcription factor, essential for normal thyroid function and healthy

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conditions. This factor has a diagnostic value in thyroid cancer. PTCS2 has several isoforms and exhibits low expression levels in thyroid cancer (Zhu et al., 2016; Statello et al., 2021; Carlevaro-Fita et al., 2020; Wang et al., 2017). Therefore, *lncRNA PTCSC2* is a candidate possibly playing a role in this cancer, though related mechanisms have remained unclear. The IncRNAPTSC2 and *FOXE1* genes are located on one chromosome near each other.

FOXE1 methylation has been shown to participate in CRC and is considered a biomarker (He et al., 2015; FOXE1 et al., 2015). The emergence of drug resistance to chemotherapy has increased interest in applying natural products such as antimicrobial peptides (AMPs) (Chauhan et al. 2021). AMPs have exhibited antimicrobial and anticancer traits (Liu et al. 2015; Hoskin et al., 2008; Yu et al., 2016; Chu et al., 2015). The LL37 AMP is an alpha-helix peptide with proper structural and functional characteristics. This study assessed *FOXE1* and lncRNA PTCSC 2 gene expression in the CRC and normal cells, and the effect of LL37 AMP was investigated.

Materials and Methods

LL37 AMP

The LL37 AMP was purchased from GenScript USA, Inc. The structural analysis and practical anticipation were conducted using the Hierarchical Neural Network (HNN) software.

Cell Culture

Various CRC cell lines (HCT-116, HT29, WiDr, and SW742) were cultured in the DMEM medium as previously described (Alghanim et al., 2020). Moreover, the NCM460 normal colon epithelial cell line was sophisticated as the control. After 24 hours of incubation, the cells were lysed, and RNA was extracted to evaluate the expression levels of *FOXE1* and lncRNA PTCSC 2 genes for comparison between CRC and normal cells.

The effect of LL37 on Cells

Various concentrations ranging from (5-400) µg/mL of LL37 AMP were prepared, added to cell cultures, and incubated for (24 and 48) hours. The MTT (Sigma Aldrich, Germany) test was implemented to verify nontoxic levels of LL37 and its effects on CRC cells. The cells' viability was measured using the ELISA (Biotek, USA) reader at 570 nm. The following is a formula evaluating the percentage of [absorbance of treated sample/absorbance of control] as previously described (Alghanimi et al., 2020).

FOXE1 and lncRNA PTCSC 2 Genes Expression

A nontoxic level of 30 µg/mL of LL37 was considered for adding to cell lines to investigate its effect on gene expression. The RNA extraction and cDNA synthesis were performed as previously mentioned (Alghanimi et al., 2020).

The *FOXE1* and lncRNA PTCSC 2 genes' specific primer sequences have been shown in Table 1. The Real-time quantitative PCR (RT-qPCR) technique was performed considering 40 cycles and the gene expression levels were calculated using this formula: the 2^(-ΔΔCt) method [where: ΔCT = CT (target)-CT (control)].

Statistical Analysis

The comparison of *FOXE1* and lncRNA PTCSC 2 gene expression was done using various tests in the SPSS version 22, such as the Shapiro test, t-test, one-way analysis of variance (ANOVA), and Pearson test. A p-value of < 0.05 was considered a significant result.

Results

Cell Cytotoxicity

At 24 hours, the (50 and 90) % lethal concentrations (LC50 and LC90, respectively) of LL37 against NCM460 normal cells were (640 and > 640) g/mL. Furthermore, as shown in Figure 1, these values at 48 hours included (160 and > 640) g/mL, respectively.

Growth Inhibitory Against Cancer Cells During (24 – 72) hours

After 24 hours of treatment, the LC50 and LC90 of LL37 AMP against CRC cell lines included (20 and 200) µg/mL, respectively, which were significantly lower than those against normal cells ($p < 0.05$). The LC50 and LC90 of the LL37 at 48 hours. included (20 and 50) µg/mL.

These values at 72 hours of exposure included (~10 and 40) µg/mL, respectively; highlighting high anticancer potential (Figures 2, 3, and 4). Therefore, the anticancer effect of LL37 was increased by enhancement of concentration and time.

Gene Expression

Before exposure to the LL37 AMP, *FOXE1* gene expression was significantly higher in CRC cells compared to that of normal cells (NCM460 and HaCaT). Moreover, the expression of the PTCSC 2 gene was not significantly different between CRC and normal cells (Figures 5 and 6).

The LL37 AMP significantly decreased *FOXE1* gene expression by 1.95-fold in CRC cells ($p < 0.001$).

Table 1. The Sequence of Primers for Each Gene

Gene	Sequence: 5'-3'	Annealing T (°C)	Reference
FOXE1	F: GTAACCAGAGGGCAGCGTAG	61	This study
	R: GCGTAAAAAGGCCCGAGTTC		
GAPDH	F: GAAGGTGAAGGTCGGAGTC	60	
	R: GAAGATGGTGATGGGATTTC		
PTCSC2	F: CCAGTCTGCAAATTCCCAAGC	60	
	R: AGGCATCCCACTCTTCCTTG		

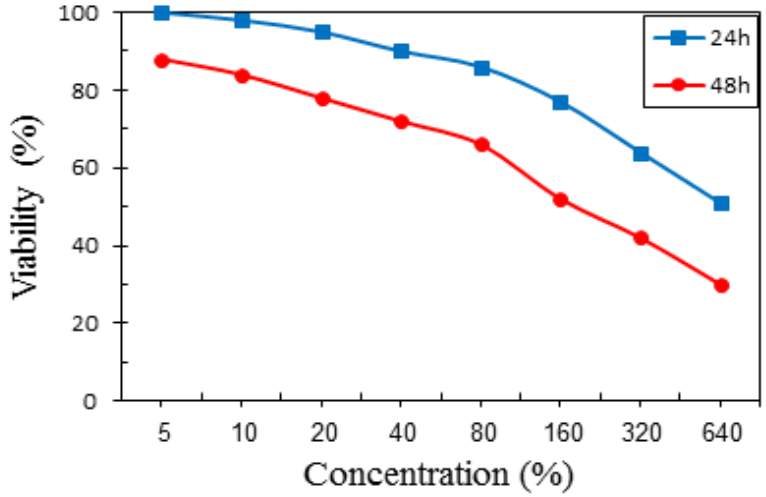

Figure 1. The MTT Test of LL37 AMP against NCM460 Normal Cells at (24 and 48) hours.

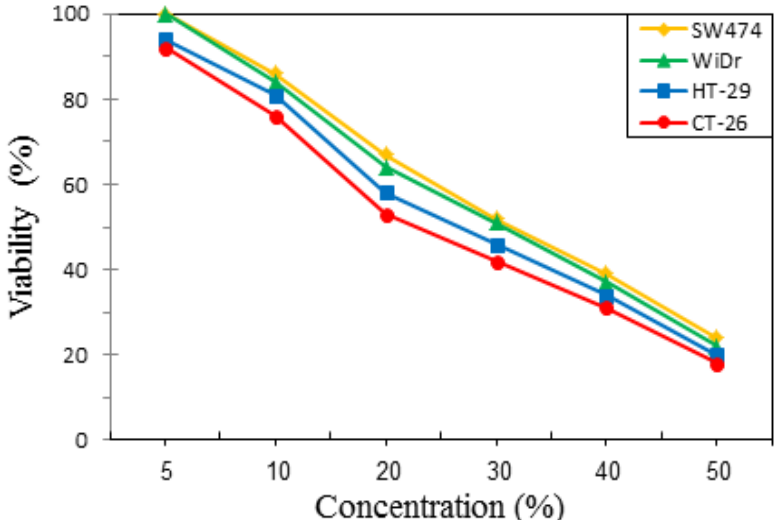

Figure 2. The LL37 Anticancer Effect at 24 hours.

However, the expression of the PTCSC2 gene decreased 0.2-fold, which was non-significant ($p = 0.177$). There was no significant difference among cell lines regarding the expression of each gene.

Discussion

There are several mechanisms originating from genetic and epigenetic factors that evoke the progression of cancer. Regulatory mechanisms and responses to them

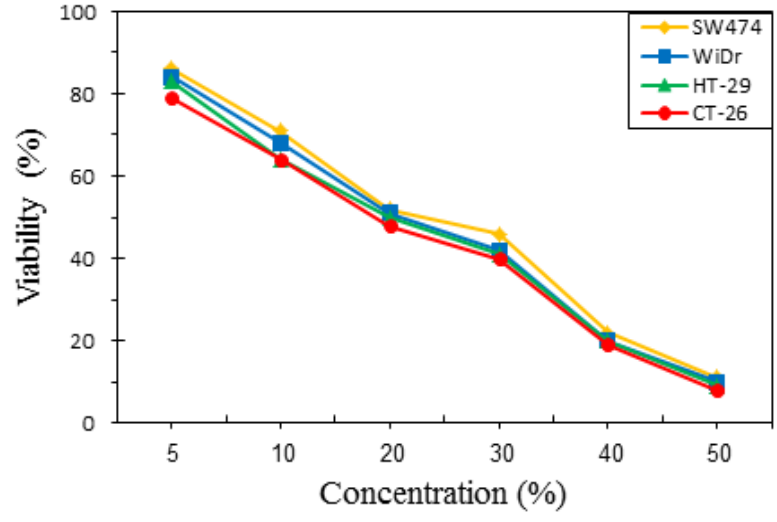

Figure 3. The LL37 Anticancer Effect at 48 hours.

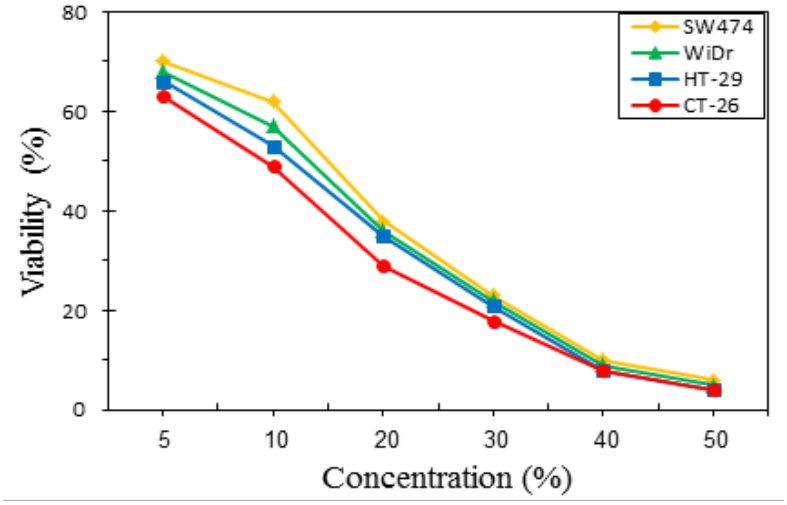

Figure 4. The LL37 Anticancer Effect at 48 hours

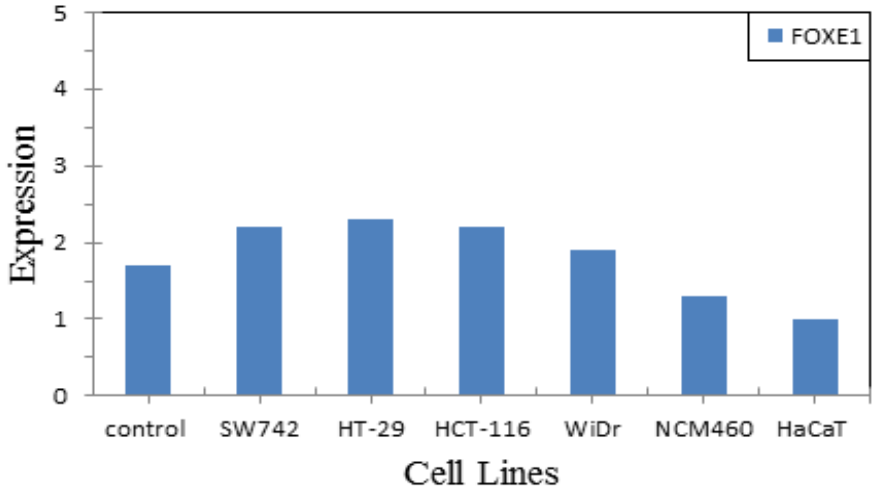

Figure 5. The Comparison of FOXE1 Gene Expression between CRC and Normal Cells

are also vital to controlling the cells' growth (D'asheesh et al., 2021; Graham et al., 2017; Chen et al., 2019; Wu et al., 2021; Ding et al., 2014). Treatment of cancers using chemotherapy has not always been successful. Various AMPs have been demonstrated to exert low toxicity against normal cells while conferring anticancer effects (Chauhan et al., 2021; Liu et al., 2015; Hoskin et al. 2008; Yu et al., 2016; Chu et al., 2015). Cancer cell membranes contain a negative charge which acts as proper targets for cationic peptides, causing cell death via various

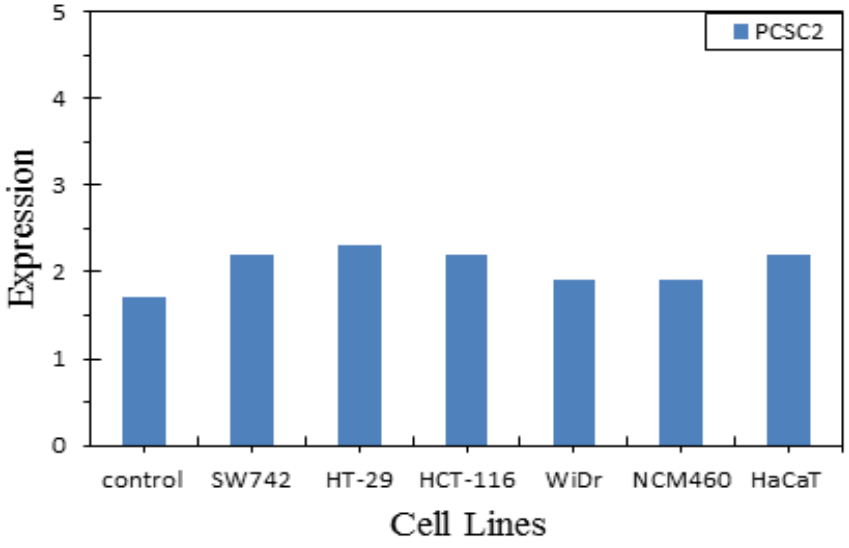

Figure 6. The Comparison of PTCSC2 Gene Expression between CRC and Normal Cells

mechanisms (Chu et al., 2015). The AMPs have enough tolerance and their production does not require high costs (Liu et al. 2015; Hoskin et al. 2008, Yu et al. 2016; Chu et al., 2015). Alpha-helix AMPs have exhibited more efficient antimicrobial and anticancer effects (Yu et al., 2016, Chu et al., 2015; Alghanimi et al., 2020).

AMPs can be modified or combined with other compounds to improve or enhance their effects (Chu et al., 2015; Alghanimi et al., 2020). The LL37 AMP secreted in human respiratory and reproductive systems and skin has exerted wide effects (Liu et al., 2015; Hoskin et al., 2008; Yu et al., 2016; Chu et al., 2015).

In this study, the LL37 AMP had anticancer effects against various CRC lines at 24 hours (200 µg/mL), 48 hours (50 µg/mL), and 72 hours (40 µg/mL), while higher concentrations were nontoxic against NCM460 normal cells (160 µg/mL after 24 hours and 640 µg/mL after 48 hours).

Hence, the effect of LL37 AMP against CRC cells was significantly higher ($p < 0.0001$). Similar to our findings, L-K6, VmCT1, magainin 2 (12 µg/mL) and related analogs, and CA-MA-2 cationic peptide (20 µg/mL have exhibited significantly higher anticancer effects but low cytotoxicity against normal cells (200 and 100) µg/mL, respectively (Saleh et al., 2022; Emelianova et al., 2018; Wang et al., 2017). AMPs have conferred their effects through various mechanisms such as apoptosis, necrosis, and membrane disturbance as observed regarding Cecropin B (silk moth), Buforin IIb (frog), Alpha-defensin-1 (human), and lactoferricin B (bovine) (Wang et al., 2017, Zandsalimi et al., 2020; Tornesello et al., 2020; Jin et al., 2019; Tsai et al., 2015).

In our survey, before exposure to the LL37 AMP, the *FOXE1* gene expression was significantly higher in CRC cells compared to that of normal cells (NCM460 and HaCaT). However, the expression of the PTCSC 2 gene was not significantly different between CRC and normal cells. Although the mechanisms of the PTCSC 2 gene in papillary thyroid cancer (PTC) are unknown, myosin (MYH9) regulates both genes' expression by binding to the promoter region (He et al., 2015; FOXE1 et al., 2021). Higher expression of the *FOXE1* gene in our study possibly highlights lower regulatory effects from MYH9 or a lack of sufficient response. In a study by (He et al., 2015), the PTCSC2 gene expression was lower in cancer cells. (Melotte et al. 2015) discovered that methylation in the *FOXE1* gene inhibits the binding and effect of regulatory factors in CRC cells.

They also proposed that this gene product in the bloodstream could be a biomarker of CRC. We also found that *FOXE1* can be considered a biomarker of CRC. Fouad et al. did another study that showed that high expression of the *FOXE1* gene is linked to advanced stages of cancer, heart attacks, getting older, and metastases. The *FOXE1* gene promotes development, metastasis, and PTC progression (Fouad et al., 2018; Ma et al., 2019). The molecular docking analysis of the CXCL1 target protein in CRC using MREEKKERKRD and MVQGAKRGGRLHRV AMPs revealed their binding and anticancer effects of them (Kumar et al., 2021).

The LL37 AMP caused a significant decrease in the *FOXE1* gene expression by 1.95-fold in CRC cells ($p < 0.001$). However, the expression of the PTCSC2 gene decreased 0.2-fold, which was non-significant ($p = 0.177$). There was no significant difference among CRC cell lines regarding the expression of each gene, and the mean level was considered for comparison. The limitations of our study mainly included the low number of genes and AMPs evaluated; a lack of in vivo studies; and also, the combination of LL37 with other compounds and anticancer drugs.

In conclusion, the *FOXE1* gene can be considered a biomarker of CRC development. However, the PTCSC2 gene was not significantly different between CRC and normal cells. The effects of LL37 AMP against CRC cells were time and dose-dependent and exerted non-toxic effects against normal cells at significantly higher concentrations. The expression of *FOXE1* but not the PTCSC2 gene was significantly affected by the LL37 AMP. Future studies are warranted to evaluate a higher number of cancer-related genes and AMPs, in vivo studies, and also the combination of LL37 with other compounds and anticancer drugs.

Author Contribution Statement

Conceptualization, Mustafa Nuhad Al-Darraji, Mohammed Rasheed, Abdolmajid Ghasemian; methodology, Mohammed Rasheed, Saade abdalkareem Jasim; investigation, Mustafa Nuhad Al-Darraji, Saade abdalkareem Jasim, Omer Dhia Aldeen Salah Aldeen, Abdolmajid Ghasemian, Mohammed Rasheed; writing-original draft preparation, Mustafa Nuhad Al-Darraji, Saade abdalkareem Jasim; writing-review and editing, Omer Dhia Aldeen Salah Aldeen, Abdolmajid Ghasemian, Mohammed Rasheed; funding acquisition, Mustafa Nuhad Al-Darraji, Abdolmajid Ghasemian, Mohammed Rasheed.

All authors have read and agreed to the published version of the manuscript.

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Ethics approval and consent to participate

This study was approved by the ethics committee of Al-Maarif University. All the authors had written consent to participate (those subjects without such consent had been excluded from the study).

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Conflict of interest

None.

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