RESEARCH ARTICLE

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Staphylococcus aureus alpha-toxin gene lead to changes in the expression of apoptosis-related genes in PC3 cell line

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Article info:
Received: 10 June 2020
Accepted: 4 July 2020

ABSTRACT

Prostate cancer is the most frequent tumor malignancy around the world which accounts for about 10% of cancer-related deaths. α-toxin is one of the *Staphylococcus aureus* pore-forming toxins affecting the expression of apoptotic-related genes. The aim of this research is to investigate the effects of *S. aureus* α-toxin on the expression of BCL2, BAX, and FAS in PC3 cell lines. In this study, PC3 cells were transfected with the pcDNA3.1(+)-α-toxin or empty pcDNA3.1(+) plasmids using Lipofectamine 2000. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Then, the mRNA expression level of BCL2, BAX, and FAS was measured by qRT-PCR method. Our findings indicated that α-toxin of *S. aureus* markedly altered the expression level of the apoptotic-related gene of the PC3 cell line. The mRNA expression of BAX and FAS genes increased statistically significant in α-toxin treated PC3 cells compared with the control group. On the contrary, BCL2 mRNA expression showed a significant decrease compared with PC3 cells transformed by empty pcDNA3.1(+) plasmid. The present research suggested that α-toxin may be a tumor suppressor in prostate cancer. This toxin may be a potential therapeutic target for prostate cancer.

Key words: *S. aureus*; *α*-toxin; PC3; prostate cancer

Introduction

Prostate cancer (PCa) is the most common malignancy in the male population worldwide and can cause about 10% of cancer-related deaths. Also, PCa is the third-leading cause of cancer death in men and hence can threaten long-term public health (Litwin & Tan, 2017; Nunes-Xavier et al., 2019). Furthermore, this cancer globally is the fifth most common cause of cancer death and estimated as 366,000 deaths in 1.6 million men diagnosed each year. The established risk factors for total prostate cancer incidence are limited to older age, African- American race, and positive family history of prostate cancer (Pernar et al., 2018). In recent years, the main risk factor for prostate cancer has been age, as the median age at diagnosis was 68, and the median age at death was 80. The prostatic hyperplasia is common in men over 70 years of age (about 95%). Due to various therapeutic methods like surgery, radiation therapy, and androgen deprivation therapy, the survival rate of PCa patients has been increased (Valkenburg & Williams, 2011).

There are various options for the screening of this disease. After suspicion, the standard diagnostic test for this tumor is tissue biopsy. Meanwhile, the distinction and characterization of this cancer have become increasingly precise through improved risk stratification and advances in magnetic resonance imaging, as well as from the emergence of biomarkers (Litwin & Tan, 2017). Despite many recent advances in the PCa therapy, this disease remains incurable. There are various treatment options for this cancer such as hormone therapies abiraterone, enzalutamide, and androgen, taxane-based chemotherapy, and treatments such as radium-223 and sipuleucel-T (Sumanasuriya & De Bono, 2018). Nowadays, there are some species of bacteria that are being used against cancer. The bacterial properties that are thought to be important factors in determining anti-tumor effectiveness are their genetic background, their species and their infectious behavior within the tumor microenvironment (Kramer et al., 2018). For the first time, William Bradley Coley, an American bone surgeon and cancer researcher, treated his cancer patients with streptococcal active cultures. Coley produced a variety of "anti-tumor vaccines" mixing heat-killed bacteria, combining Staphylococcus pyogenes with Serratia marcescens which later became known as "Coley's toxins". It was applied to patients with sarcomas, carcinomas, lymphomas, melanomas, and myelomas (Kramer et al., 2018).

Staphylococcus aureus is a major human pathogen that generates a wide range of toxins, thus causing different kinds

of disease symptoms. It is the leading cause of bacteremia, infective endocarditis (IE), and can cause, skin and soft tissue, osteoarticular, pleuropulmonary, and device-related infections. *S. aureus* can express virulence factors and different resistance mechanisms, allowing it to escape host natural defenses (Balaban & Rasooly, 2000; Kramer et al., 2018). The efficiency of *S. aureus* as a pathogen can be accredited to its virulence factors resource which its secreted toxins play an important role. Hence, its toxin that can activate apoptosis could serve as a potential tool for cancer treatment. The main *S. aureus* toxins can be divided into three most important groups; the superantigens (SAgs), pore-forming toxins (PFTs), and exfoliative toxins (ETs). Pore-forming toxins can be further separated into four types; Hemolysin (Hla or alpha-toxin), Hemolysin-b, leukotoxins, and phenol-soluble modulins (PSMs) (Oliveira et al., 2018; Zhang et al., 2017). One kind of *S. aureus* toxin secreted by 95% of clinical *S. aureus* strains is a 33 kDa polypeptide called α-toxin. It is a beta-barrel forming toxin secreted as a water-soluble monomer. Its bonding capability and oligomerization into a heptameric structure on the host cell membrane make this toxin dangerous. This toxin affects a wide range of human cell types, including endothelial cells, epithelial cells, T cells, macrophages, and monocytes (Oliveira et al., 2018). Studies have reported that T cells purified from peripheral blood can undergo apoptosis by stimulating α-toxin. Also, another research showed that α-toxin can lead to apoptosis in bladder cancer cell line (Zhang et al., 2017).

One of the main prostate cancer cell lines is PC3. It was derived from bone metastasis of a grade IV prostate adenocarcinoma in 1979 (Costa et al., 2018). PC3 cells do not express prostate-specific antigen (PSA) and androgen receptor (AR) and are androgen-independent. They exhibit very aggressive behavior which is not similar to typical clinical cases of PCs (Tai et al., 2011).. Human gene BCL2 is a proto-oncogene, located on the chromosomal region 18q21.3. This gene is mostly expressed in fetal tissues and codes for an anti-apoptotic protein (26 kDa) which can prevent the release of cytochrome c from the mitochondria, thus inhibiting the activation of caspases and finally obstructing apoptosis. BAX (BCL2 associated X protein) gene is placed on the human genomic region 19q13.3-q13.4. In cancerous samples, BAX gene expression was considerably reduced at the mRNA and protein levels. Further, another study indicated that BAX overexpression has inhibitory effects on cell growth, and hence it probably induces apoptosis in cells (Korbakis & Scorilas, 2012). The first apoptosis signal (FAS) gene triggers the apoptosis pathway and plays a major role in tumor growth. FAS gene is located on chromosome 10q23. FAS is also known as CD95 or APO-1 is a cell surface protein belonging to a member of the tumor necrosis factor receptor (TNFR) family (Jalali et al., 2018). The aim of the present study is to investigate the expression of apoptotic related genes such as BCL2, BAX, and FAS in PC3 prostate cancer cell line transfected with a recombinant vector contain the gene which encoded the α-toxin of *S. aureus*.

Materials and Methods

Construction of recombinant plasmid

The backbone for the construction of recombinant plasmid was the mammalian expression vector pcDNA3.1(+) (Invitrogen). In this vector encoding, recombinant proteins are expressed under the control of the CMV promoter. The 963 bp α-toxin genes from *S. aureus* was cloned into pcDNA3.1(+) between BamHI and EcoRV restriction sites. *E. coli* strain TOP10F competent cells were transformed by plasmids using the heat shock method. Plasmid extraction was performed using the Bioneer Plasmid Kit (Bioneer, South Korea). The recombinant plasmid was confirmed by PCR amplification, double digestion with appropriate restriction enzymes, and DNA sequencing.

Cell culture and transfection

The human prostate cancer cell line (PC3) was obtained from the national cell bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin-streptomycin and 1% glutamine. All the cells were grown and maintained at 37℃ under a humidified atmosphere of 5% CO_2.

PC3 cells were transfected in a 6-well plate by pcDNA3.1(+)-*α-toxin* or the pcDNA3.1(+) plasmid (control group) using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Four hours after transfection the media was then replaced with fresh medium containing 600 mg/L G418 and 10% FBS for 3 weeks for the stable cell line selection.

Total RNA extraction and reverse transcription-PCR

Total RNA was extracted from transfected PC3 cells with TRIzol reagent (Invitrogen, USA) according to manufacturer instructions and the RNA content was measured spectrophotometrically at 260 nm. Approximately 2μg of total RNA was reverse transcribed into first strand cDNA using random primers. The cDNA samples were stored at -80°C until use. Eukaryotic expression of *S. aureus* α-toxin mRNA in PC3 cells was detected by the RT-PCR method using α-toxin specific primers (Table 1). PCR was performed in 20 μL reaction volume containing 1 μL of cDNA, 2 mM $MgCl_2$, 1 pmol of forward and reverse primers, and 0.25 U Taq DNA polymerase (New England BioLabs, USA). The following amplification program was used: 94°C for 5 min, 30 cycles of 94°C for 45 s, 64°C for 45 s and 72°C for 1 min, followed by 72°C for 7 min.

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DOI: 10.69085/jbb20202001

Table 1. *Sequences of primers used in this study.*

Genes	Forward and Reverse	Sequences	Annealing temp (ºC)	Product length (bp)
α-toxin	Forward	5'-ACACTGCTGCTCGGCTCCATTC-3'	66	227
	Reverse	5'-ATGGTGCCCTTTGTGCGAATC-3'		
BCL2	Forward	5'-GACGACTTCTCCCGCCGCTAC-3'	65	245
	Reverse	5'-CGGTTCAGGTACTCAGTCATCCAC-5'		
BAX	Forward	5'-AGGTCTTTTTCCGAGTGGCAGC-3'	65	234
	Reverse	5'-GCGTCCCAAAGTAGGAGAGGAG-3'		
FAS	Forward	5'-CAATTCTGCCATAAGCCCTGTC-3'	64	163
	Reverse	5'-GTCCTTCATCACACAATCTACATCTTC-3'		
GAPDH	Forward	5'-GCCAAAAGGGTCATCATCTCTGC-3'	64	183
	Reverse	5'-GGTCACGAGTCCTTCCACGATAC-3'		

The amplification products were separated by electrophoresis in 1.5% agarose gels and visualized by staining with ethidium bromide.

qRT-PCR

Quantitative RT-PCR was done using the SYBR®Premix Ex TaqTM II kit (TaKaRa, Japan) and specific primers (Table 1). The mRNA expression of BCL2, BAX, and FAS were evaluated with a Rotor-Gene 6000 Machine (Corbett Life Science, Concorde, NSW, Australia). GAPDH mRNA was used as an internal control. PCR cycling conditions were: the first step at 95 °C for 3 min, followed by 40 cycles at 95°C for 15 s, 64°C for 20 s and 72°C for 20 s for gene expressions assay. To check for the primers' specificity, melting curves were generated with temperatures ranging from 55°C to 95°C after the 40 cycles. To verify reaction efficiencies, for each primer set, standard curves were prepared using data from serially diluted samples. A non-template control (NTC) was run with every assay, and all determinations were performed in duplicates to achieve reproducibility. The interpretation of the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

GraphPad Prism (GraphPad Software, USA) was used for data analysis. Statistical comparisons were performed using the Student's t-test of at least three independent experiments. The differences with a p-value less than 0.05 were considered as the statistical significance level.

Results

Evaluation of recombinant plasmid

To confirm the pCDNA3.1(+)-*α-toxin*, PCR and *BamH*I/*Eco*RV restriction enzymatic double digestion were used. The products of PCR and this digestion were analyzed by agarose gel electrophoresis. Accordingly, a fragment of 227 bp was observed as a PCR product on 1% agarose gel. Also, two DNA bands with 5.4 kb and 963 bp were obtained with *BamH*I/*Eco*RV digestion for pCDNA3.1(+) vector and *α-toxin* insert, respectively. DNA sequencing further confirmed the pCDNA3.1(+)-*α-toxin* recombinant vector (data – not shown). Also, Transfection efficiency calculated by determining the number of cells that exhibit transfected DNA over the total number of cells in the sample. The number of cells calculated under the microscope with G418.

Expression of α-toxin mRNA in PC3 cells

The 227 bp fragment corresponding to the *α-toxin* cDNA was amplified by RT-PCR. This band was presented only from pCDNA3.1(+)-*α-toxin* transfected PC3 cells, but not from pCDNA3.1(+)-transfected cells (data – not shown).

Quantitative assessment of the mRNA expression levels of BCL2, FAS and BAX

A specific qPCR was used to evaluate the mRNA expression levels of BCL2, BAX, and FAS genes in PC3 cells transfected with pCDNA3.1(+)-*α-toxin* and pCDNA3.1(+)-transfected cells. The specific qPCR amplicons were evidenced by a peak in the melting curve analysis and the detection of a single distinctive band in agarose gel electrophoresis for each gene. The comparative study of the mRNA levels of BCL2, FAS, and BAX between the *α-toxin*-treated cells and control group enabled the identification of important differential expression patterns.

The mRNA levels of BAX and FAS presented an ascending trend in *α-toxin*-receiving PC3 cells compared with the control group (Figure 1) which was statistically significant ($P< 0.05$).

Additionally, the BCL2 expression was significantly reduced (2-fold) in the *α-toxin*-treated PC3 cells compared with PC3 cells transformed by empty pcDNA3.1(+) plasmid ($P< 0.05$).

Discussion

Following lung cancer, the second reason for cancer-related deaths is prostate cancer (Ashtiani et al., 2017). Novel drugs, drug sequences, and combinations are known as recent studies on prostate cancer therapy in recent years. As the latest

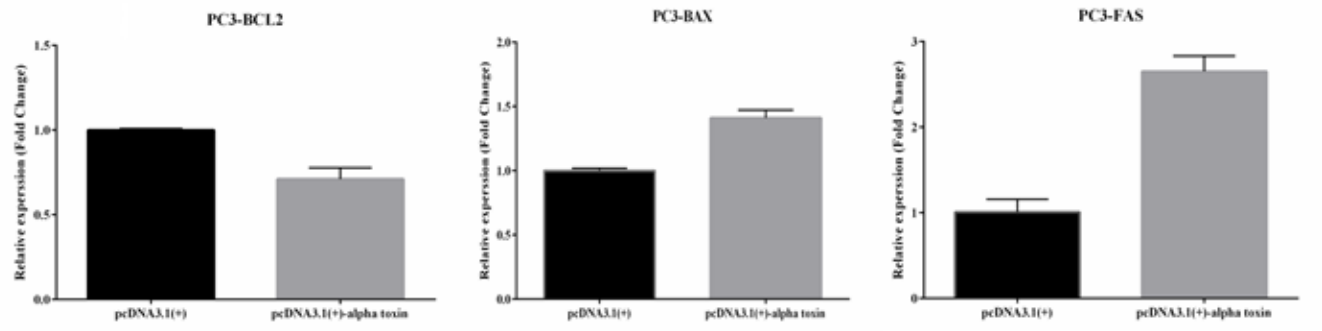

Figure 1. *mRNA expression levels of BCL-2, BAX and FAS in α-toxin-treated PC3 cells compared with control group.*

approval, we can mention abiraterone acetate which targets androgen receptor (AR) signaling. Adding abiraterone acetate to androgen deprivation therapy to achieve complete androgen blockade has witnessed extremely advantageous for the treatment of advanced prostate cancer (Nevedomskaya et al., 2018). Cancer mono-therapy approach (using a single drug for cancer treatment) is still a usual treatment assay for various types of cancer. Not only this method is known as a conventional method but it also generally deemed less effective (Bayat Mokhtari et al., 2017). On the other hand, cancers find the ability to resistance to traditional therapies, and by increasing the prevalence of these drug-resistant trends, the treatment of cancer become more complicated (Housman et al., 2014).

The suggested treatments for prostate cancer such as chemotherapy and radiotherapy induce apoptosis in cells. Hence, the degradation of tumors by including apoptosis is the most common mechanism of cancer therapeutics (Amiri et al., 2018). Thus, many studies are ongoing to promote cell apoptosis in cancer cells. BAX, BCL-2, and FAS genes play a key role and are the main candidates for the onset or inhibition of apoptosis genetically. BCL-2, which is known as an anti-apoptotic member of a family with the same name, is one of the first regulators of apoptosis. It suppresses apoptosis by blocking the escape of cytochrome c from the mitochondria to stop the activation of caspases. It has also been related to androgen-independent prostate adenocarcinomas (Amiri et al., 2018; Sihombing et al., 2017). BAX gene is also one of the most important and active members of the apoptotic group. Signals of stress or damages induce oligomerization of BAX monomers and lead to the release of pro-apoptotic proteins and activation of some caspases. In addition, this gene can release cytochromes by creating holes in the mitochondria (Amiri et al., 2018). FAS gene has also an important effect on tumor growth and stimulates the apoptosis pathway.

In the current study, given the high toxicity of α-toxin for eukaryotic cells, the expression and transmission of this gene in cancer cells were examined. The findings showed that the expression of BAX gene such as FAS gene increased significantly ($P < 0.05$) in PC3 cancer cells containing an α-toxin encoding gene. To expound BAX and FAS over-expression in the presence of an α-toxin gene in PC3 treated cells, it can be mentioned that the toxicity of α-toxin can initiate apoptotic processes and induce inhibition of anti-apoptotic pathways. Thus, by considering the role of BAX and FAS genes in apoptosis, the over-expression of these genes by α-toxin can be related to the beginning of cell death in cancer cells.

Meanwhile, there are many studies conducted on the effect of *S. aureus* toxins on the expression of mentioned apoptotic genes. The paper, written by Zhang et al. (2017), reported various effects of *S. aureus* toxins on gene expression such as BAX, BCL-2, and FAS. In detail, overexpression of BCL-2 could decrease the induction of apoptosis by α-toxin in Jurkat T cells, but FAS does not have this ability (Zhang et al., 2017). This can suggest that the decrease in BCL-2 mRNA expression in α-toxin-treated PC3 cells is due to the apoptosis induced by α-toxin. Further, the overexpression of the FAS gene could demonstrate the same result. In another research, investigation of the expression of the genes involved in apoptosis indicated that staphylococcal enterotoxin B could reduce the expression of BCL-2 by inducing more apoptosis in liver cells. Both enterotoxin B and α-toxin are bacterial toxins, reduction of the mRNA expression of BCL-2 in the current study could be caused by α-toxin-induced apoptosis.

Hence, as mentioned above, BAX pro-apoptotic activities which include releasing cytochromes and activation of some caspases can occur via apoptosis cascade by α-toxin.

Elsewhere, Amiri et al. in 2018 found that ethanol baneh extract decreased the expression of BCL2 and increased the expression of Bax gene, while also increasing caspase3 concentration (Amiri et al., 2018). The results of this research also showed that the concentration of caspase3 in PC3 cells was elevated by baneh extract. Thus, ethanol baneh skin extract could induce apoptosis in PC 3 cells through the modulation of expression of BAX and BCL-2 genes. In the

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DOI: 10.69085/jbb20202001

current study similar to ethanol baneh, α-toxin also led to overexpression of BAX gene and reduction of BCL-2 gene. In addition, by increasing the BAX gene expression, both ethanol baneh and α-toxin can cause an increase in the concentration of some caspases.

The effect of imatinib on BCL-2 gene was investigated by Shandiz et al. (2016). They found that imatinib leads to the down-regulation of BCL-2 expression in prostate cancer PC3 cell lines. Similar to imatinib, the current study findings showed that α-toxin can lead to the down-regulation of BCL-2 in PC3 treated cells. Thus, these two compounds have a similar effect on the expression of the BCL-2 gene.

Conclusion

In conclusion, based on the findings obtained from our study, α-toxin induced the down-regulation of BCL-2 gene expression and up-regulation of BAX gene in prostate cancer PC-3 cells. Hence, it may be a good candidate as an inhibitor of the growth of cancer cells *in vivo* and for the treatment of prostate cancer. Overall, the outcomes showed here warrant further investigation on other prostate cancer cell lines such as animal tumor models and subsequent clinical studies.

Acknowledgements

The project resulted from Arezoo Sadeghi M. Sc thesis and we would like thanks from the deputy of research of Shahrekord Branch, Islamic Azad University. Also, authors of this article are thankful to the staff of Biotechnology Research Center, Shahrekord Branch, Islamic Azad University.

References

Amiri M, Nasrollahi F, Barghi S, Ebrahimi N, Rajizadeh A, Dehghan Nayyeri N, Kazerouni F, Rahimipour A, Namaki S, Ahmadi H. 2018. The Effect of Ethanol Baneh Skin Extract on the Expressions of Bcl - 2, Bax, and Caspase - 3 Concentration in Human Prostate Cancer pc3 Cells. *Int. J. Cancer. Manag. Kowsar*., 11(3): 1-8.

Ashtiani M, Nabatchian F, Galavi HR, Saravani R, Farajian Mashhadi F, Salimi S. 2017. Effect of Achillea wilhelmsii extract on expression of the human telomerase reverse transcriptase mRNA in the PC3 prostate cancer cell line. *Biomed. Reports*., 7(3): 251-256.

Balaban N, Rasooly A. 2000. Staphylococcal enterotoxins. *Int. J. Food. Microbiol.*, 61(1): 1–10.

Bayat Mokhtari R, Homayouni TS, Baluch N, Morgatskaya E, Kumar S, Das B, Yeger H. 2017. Combination therapy in combating cancer. *Oncotarget*., 8(23): 38022-38043.

Costa J, Pereira R, Oliveira J, Alves Â, Marques-Magalhães Â, Frutuoso A, Leal C, Barros N, Fernandes R, Queiroz Almeida D, Barreiro M, Barros A, Sousa M, Sá R. 2018. Structural and molecular analysis of the cancer prostate cell line PC3: Oocyte zona pellucida glycoproteins. *Tissue. Cell*., 55: 91-106.

Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, Sarkar S. 2014. Drug Resistance in Cancer: An Overview. *Cancers (Basel)*., 6(3): 1769–1792.

Jalali SA, Homaei Shandiz F, Tavakol Afshari J, Davarpanah Tanha Ghochan M, Nikpoor AR, Mohammadi M. 2018. Status of FAS and FAS Ligand Gene Polymorphisms in Patients with Breast Cancer in Northeastern IRAN. *Reports. Biochem. Mol. Biol.*, 7(1): 23-29.

Korbakis D, Scorilas A. 2012. Quantitative expression analysis of the apoptosis-related genes BCL2, BAX and BCL2L12 in gastric adenocarcinoma cells following treatment with the anticancer drugs cisplatin, etoposide and taxol. *Tumor. Biol.*, 33(3): 865-875.

Kramer MG, Masner M, Ferreira FA, Hoffman RM. 2018. Bacterial Therapy of Cancer: Promises, Limitations, and Insights for Future Directions. *Front. Microbiol.*, 9: 16.

Litwin MS, Tan HJ. 2017. The Diagnosis and Treatment of Prostate Cancer. *JAMA*, 317(24): 2532.

Nevedomskaya E, Baumgart SJ, Haendler B. 2018. Recent Advances in Prostate Cancer Treatment and Drug Discovery. *Int. J. Mol. Sci*., 19(5): 1359.

Nunes-Xavier CE, Mingo J, López JI, Pulido R. 2019. The role of protein tyrosine phosphatases in prostate cancer biology. *Biochim. Biophys. Acta. Mol. Cell Res.*, 1866(1): 102–113.

Oliveira D, Borges A, Simões M. 2018. *Staphylococcus aureus* Toxins and Their Molecular Activity in Infectious Diseases. *Toxins (Basel).*, 10(6): 252.

Pernar CH, Ebot EM, Wilson KM, Mucci LA. 2018. The Epidemiology of Prostate Cancer. *Cold Spring Harb. Perspect. Med.*, 8(12): a030361.

Shandiz SAS, Farasati S, Saeedi B, Baghbani Arani F, Asl EA, Keshavarz Pakseresht B, Rahimi A, Assadi A, Noorbazargan H, Hesari MR, Mirzaie A. 2016. Up regulation of KAI1 gene expression and apoptosis effect of imatinib mesylate in gastric adenocarcinoma (AGS) cell line. *Asian Pacific J. Trop. Dis.*, 6(2): 120-125.

Sihombing B, Syukur S, Ibrahim S, Cong DH, Setyoheryanto D. 2017. Absence of BRCA1 185delAG, BRCA1 5382InsC and BRCA2 6174delT among Hereditary Breast Cancer Patients in North Sumatera, Indonesia. *ASRJETS*, 38(2): 152-160.

Sumanasuriya S, De Bono J. 2018. Treatment of Advanced Prostate Cancer—A Review of Current Therapies and Future Promise. *Cold Spring Harb. Perspect. Med.*, 8(6): a030635.

Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, Huang J. 2011. PC3 is a cell line characteristic of prostatic small cell carcinoma. *Prostate*., 71(15): 1668-1679.

Valkenburg KC, Williams BO. 2011. Mouse Models of Prostate Cancer. *Prostate Cancer*., 2011: 1-22.

Zhang X, Hu X, Rao X. 2017. Apoptosis induced by *Staphylococcus aureus* toxins. *Microbiol. Res.*, 205: 19-24.