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ORIGINAL ARTICLE

Screening of Anticancer and Immunomodulatory Properties of Recombinant pQE-HAS113 Clone Derived from Streptococcus Equi

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ABSTRACT

Objective: Streptococcus equi has been identified to contain the hyaluronan synthase gene (HAS), which could have a role in the cell proliferation activity. The objective of this research was to determine anticancer properties of HAS113 recombinant clone.

Methodology: The intracellular and extracellular portions of the expressed gene products of pQE-HAS113 were harvested and evaluate for anticancer properties against prostate cancer cell (PC3) and breast cancer cell (MCF-7). To evaluate anti-proliferative properties of pQE-HAS113, two assays were carried out: MTT assay, which was used for assessing percent cell inhibition and apoptosis assay, through the AO/EB stain of living, apoptotic, as well as necrotic cells. For evaluation of the immunomodulatory properties of pQE-HAS113 on RAW264.7 in terms of IL-6, IFN-γ & IL-8 expression, flow cytometry examination was done. The harvested intracellular and extracellular portions of pQE-HAS113 were tested.

Results: The results indicated that the percentage proliferation of both the prostate cancer and breast cancer cells were 2.1% and 7.2% respectively against the intracellular portions of pQE-HAS113. While in case of the extracellular portions of pQE-HAS113 the proliferation rate of both the prostate cancer and breast cancer cells were 6.5% and 6.7% respectively. The PQE-HAS113 intracellular clone gene has shown proinflammatory activity by stimulating the expression of IL-6, INF-γ, & IL-8 in the RAW264.7 macrophage cells.

Conclusion: Thus, the results from the present study indicated that the expressed product of recombinant clone of HAS 113, pQE-HAS113 has potential to be used as anti-proliferative and apoptosis inducing substance for prostate and breast cancer.

Keywords: Streptococcus equi; breast cancer; cell proliferation; apoptosis ass

INTRODUCTION

Streptococcus is a genus belongs to the phylum Firmicutes and in the Lactobacillales order. From the name itself, 'Streptos' which derived from Greek language meaning easily twisted or bent like a chain thus denote that they are arranged in a single axis and a can grow in a chains or pairs and they are spherical gram positive bacteria[1]. This bacterium can be classified through their hemolysis properties. Hemolysis reaction can be divided into three types which are alpha hemolysis, beta hemolysis and gamma hemolysis[2].

Hyaluronic acid (HA) is a polymer disaccharide with a very high molecular weight that consist of N-acetylglucosamine and glucoronic acid. HA is made at the plasma membrane and secreted into the extracellular matrix by hyaluronan synthase [3]. HA can be found in mammalian connective species as a lubricant in between joints, vitreous bodies, cartilages. Its function also involved as structure maintenance of tissues when inflammation occured. This characteristic lead to the HA to have a very high viscoelasticity and water holding function [4].

Hyaluronan synthase (HAS) is made from a phospolipid dependent integral membrane protein with eight membrane domains units which active as monomer. In mammalian, there are three types of HAS genes; HAS1, HAS2 and HAS3. Each of the isoenzymes of HAS are different in kinetic characteristics and product size [5]. With these differences, the product HA will have various expression pattern in biological or physiological roles [6]. Cancer can be divided into malignant cancer and benign cancer. Malignant cancer can be defined as growth of cell that is invasive and able to metatstasize, while benign growth in another ahand is not invasive. Name of cancer can be according to the organ where the cancer started [7]. There are mant types of cancer includes cervix, breast, colon, prostate, liver and nose cancer [8]. A treatment that is offered to cure cancer is called chemotherapy. Chemotherapy treats cancer by the delivery of anti-cancerous medications in the affected person. Chemotherapy is a general form of therapy i.e. it has capability to eradicate cancerous cells through the affected body [9, 10].

Previous research conducted by Molecular Bacteriology and Toxicology Laboratory, University of Malaya, indicated that HAS113 clone obtained from S. equi via shotgun cloning technique has the ability to heighten the wound healing properties in mice. This HA property has become our interest to find out their effect in cell proliferation on cancer cell. The objective of this resaerch was to do a prelimenary screening of hyaluronic acid effect on cancer cell line, To conduct plasmid stability and harvest the purified gene products, to determine anticancer properties from HAS113 recombinant clone, pQE-HAS113 obtained from S. equi via shotgun cloning and to evaluate the anticancer properties on prostate cancer cell (PC3) and breast cancer cell (MCF-7).

MATERIALS AND METHODS

Bacterial strains, plasmid and recombinant clone: The recombinant clone pQe32-HAS113 was obtained and maintained in Molecular Bacteriology and Toxicology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur. The recombinant clone's insert which encoding for hyaluronan synthase activity, was derived from the genome of *Streptococcus equi* via shotgun cloning method. DNA supercoiled ladder was used as molecular weight marker for clone pQe32-HAS113.

Cancer cell lines: The prostate cancer (PC3) and breast cancer cell (MCF-7) and normal WRL 68 and murine macrophage cell line (RAW 264.7) acquired from the American Type Culture collection (ATCC, Rockville, MD). Plasmid Stability and reconfirmation of recombinant clone HAS113.

Plasmid DNA extraction: Plasmid extraction was done using Faber et al. [11] and Mary et al. [12] protocol.

Digestion of recombinant DNA plasmid HAS 113 and pQE DNA Vector: The insert HAS113 and vector, which is pQE fragments is prepared by undergoing the digestion process by

using enzymes RE1 and Re2. RE1 and RE2 that was used are the Kpn-1 and BamH1 respectively. Firstly, 20.5 µl of extracted HAS113 plasmid DNA was added to 1.0 µl of RE1 and RE2 respectively and with addition of Cut Smart Buffer of 2.5µl. Then, the microcentrifuge was spined or vortex at 11,000 rpm for 60 seconds and incubated for 1 to 2 hours at room temperature. The above steps were repeated to digest the pQE vector. Ligation was done by the formulation for ligation process created by Cranenburgh et al. [13].

Transformation: From the total volume of the ligated solution, transformation process was done by adding 1-5µl of the solution into 50µl of competent *E coli* JM109. Then the mixture was combined gently and for 20 minutes was placed on ice. After that, heat shock was introduced to the culture for 5 minutes at 42ºC. 100µl of LB broth was added to the culture and was incubated about 60 to 90 minutes at room temperature.

Detection of HA in expressed clone HAS 113: About 50 µl of the aliquots was cultured on LB agar followed by overnight incubation at room temperature. It was then transferred into 100 ml LB broth prior to 48 hours incubation at 37 ºC with shaking (Thermo Scientific, MaxQ 4000). The broth culture is chilled on ice for 20 minutes before centrifugation at 3500 rpm (Thermo Scientific Sorvall ST40R), 4 ºC and 60 minutes.

Proliferation assay: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Merck, Germany) was utilized to assess the impact of cloned gene on cell proliferation [14]. Morphological assessment of apoptosis by acridine orange-ethidium bromide (AO + EB) staining was done by methodology of Chitnis et al (1996). While In vitro stimulation for intracellular cytokine production was done using methodology documented by Fransic et al. (2003) [15]. A "FACSCanto II" flow cytometer and "FACSDiva version 6.1.3" software (BD Biosciences) were used to perform the cytokine analyses.

RESULTS

Plasmid stability of recombinant clone HAS113

Plasmid extraction: Agarose gel electrophoresis analysis was performed for extracted plasmid to visu-alize the band size. The band size that showed by the gel slab under UV light estimated around 4.4 kb. Thus by reconfirming the size of the recombinant HAS113 further study can be proceeded (Figure 1a).

Digestion of HAS113 and pQE vector: Both the recombinant clone of HAS113 and pQE vector undergo digestion process first before they allowed to be ligated together. The insert of the plasmid DNA of HAS113 and the vector were treated by 2 restriction enzymes namely RE1: Kpnh1 and RE2: BamH1. Then, both insert and vector are then purified under from DNA or en-zyme as to avoid contamination. The purified compound undergoes another confirm-atory testing, which is the gel electrophoresis to confirm the presence of the purified insert and vector (Figure 1b).

Proliferation assay: The results revealed that the antiproliferative impact of the cloning gene on the proliferation of cancerous cell line (MCF-7 cell, and PC3 cell) was higher, while there was no effect on the growth of normal cell lines (WRL 68 and RAW264.7). Complete description of percentage growth of cancerous and wildtype cell lines due to the anti-proliferative effect of the cloning gene are illustrated in Table 1.

Morphological assessment of apoptosis by Acridine Orange/Ethidium Bromide Staining: Acridine orange is a vigorous pigment and stained both living and dead cells, while ethidium bromide-stained cells that undergo apoptosis only. Live cells appeared equivalently green. Initial apoptotic cells were stained green with bright spots present in the nuclei in green color. Late- apoptotic cells will also integrate ethidium bromide and thus stained orange, but then in comparison to necrotic cells appeared in red color. The staining process is done to the cells that include the normal cell as control and pQE-HAS 113 treated prostate cancer (PC3) and breast cancer cell (MCF-7) (Figure 2). Cancer cells treated pQE-HAS113 fractions, were analysed in a well-defined manner as shown in figure 3A and 3B by differences in the morphology state of the treated cells. In figure 3B2, we observe that the PC3 cells, which were treated with pQE-HAS 113 intracellular fractions, underwent early apoptosis as indicated by the presence of bright green spot, occurring because of the condensation of chromatin as well as nu-clear fragmentation. While PC3 cells without pQE-HAS 113 treatment have shown proliferative activity indicated by their green fluorescence as shown in figure 3B1. Based on the results of this study, prostate cancer cells have shown higher positive re-sult after treated with pQE-HAS113 intracellular extract, where more bright green spots cells were observed, indicating that the cell underwent early apoptotic stage. The presence of orange color in the cells indicated late apoptosis. Thus, the results from the present study indicated that pQE-HAS113 extracellular portion has shown effective IC50 percent inhibition against the prostate cancer cells in lowest concentration.

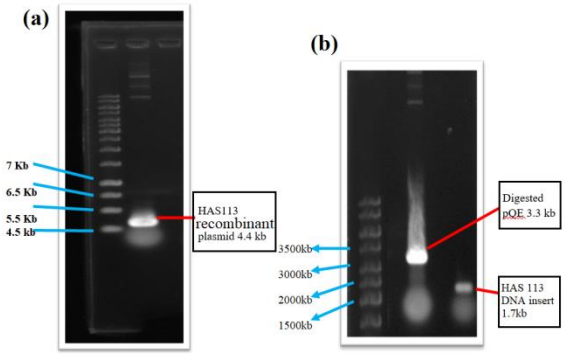

Figure 1. Agarose gel electrophoresis slab (0.7%) of plasmid DNA of recombinant of clone HAS113 reconfirmation. Lane 1: Supercoiled DNA ladder, Lane 2: HAS113 recombinant DNA plasmid (b) Double-digested HAS113 fragment with pQE vector on agarose gel electrophoresis slab (0.7%). Lane 1:1 kb DNA ladder, Lane 2: Double-digested pQE vector, Lane 3: HAS113 fragment/DNA insert.

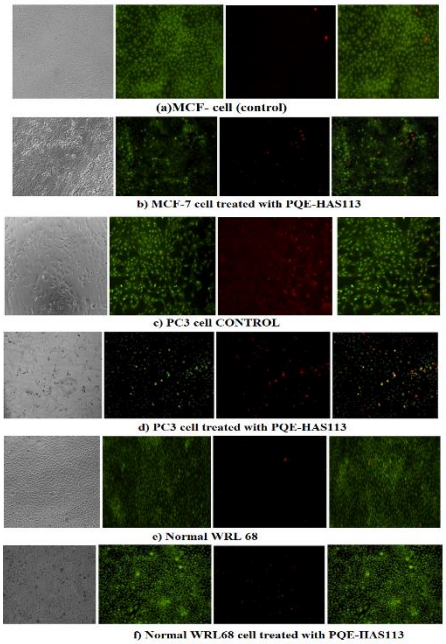

Figure 2. Morphological assessment of apoptosis in the different cancerous cell line treated with PQE-HAS113 clone gene compared with control cell. (a): MCF- cell (control, without PQE-HAS113 treatment) (b): MCF-7 cell treated with PQE-HAS113, (c): PC3 cell (control, without PQE-HAS113 treatment) , (d) : PC3 cell treated with PQE-HAS113,(e) WRL cell((control, without PQE-HAS113 treatment), (f) WRL cell treated with PQE-HAS113.

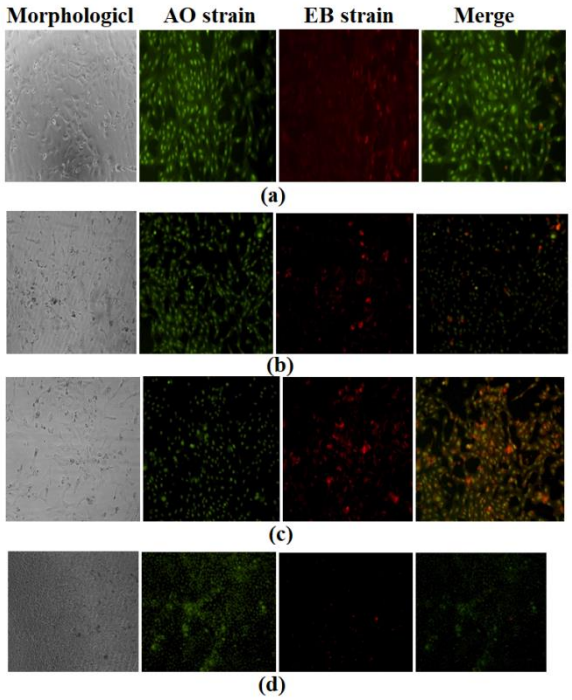

Figure 3. (a) The AO/EB stained on normal cell as control. (b) The AO/EB stained on prostate cancer cell that has been subjected to pQE-HAS 113 Intracellular extract. (c) The AO/EB stained on prostate cancer cell that has been subjected to pQE-HAS 113 extracellular extract (d) The AO/EB stained on breast cancer cell that has been subjected to pQE-HAS 113 Intracellular extract.

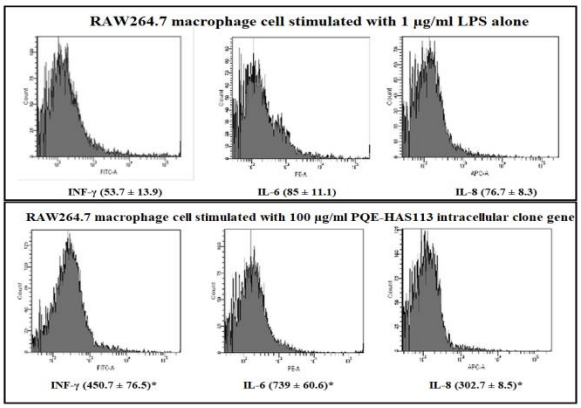

Figure 4. Effect of the 100 µg/ml PQE-HAS113 intracellular clone gene on the intracellular expression of INF-γ, IL-6 & IL-8 in the RAW264.7 macrophage cell compared with RAW264.7 macrophage cell stimulated with 1 µg/ml LPS. * Significant (P ≤ 0.05).

Flow cytometry analysis: Immunomodulatory effect on the intracellular expression of IL-6, IFN-γ and IL-8. The findings have showed significant effect (P ≤ 0.05) of the PQE-HAS113 clone gene on the immunomodulation of RAW264.7 macrophage cell in terms of intracellular-lar appearance of cytokines i.e., IL-6, IFN-γ and IL-8. Treated RAW264.7 macrophage cell with PQE-HAS113 clone gene caused increasing in the cytokine expression that revealed to the immunostimulatory effect when compared with RAW264.7 macro-phage cell stimulated with LPS only (Figure 4).

Table 1. The effect of the PQE intra and extracellular expression on the proliferation of cancer (MCF-7 CELL and PC3 cell) and normal (WRL cell and RAW 264.7 cell) cell lines.

GROUPS	MCF-7 Cell % Proliferation	PC3 Cell % Proliferation	WRL Cell % Proliferation	RAW264.7 Cell % Proliferation	Significance
PQE intracellular 25 ug/ml	7.2 ± 0.4	2.1 ± 1.7	104.0 ± 0.4	100.5 ± 0.3	(*P ≤ 0.05*)
PQE intracellular 50 ug/ml	11.8 ± 2.3	7.9 ± 3.9	124.9 ± 4.5	101.0 ± 1.4	(*P ≤ 0.05*)
PQE intracellular 100 ug/ml	15.3 ± 0.2	21.8 ± 1.5	130.2 ± 1.7	109.2 ± 1.4	(*P ≤ 0.05*)
PQE intracellular 200 ug/ml	21.3 ± 0.1	40.3 ± 5.1	144.8 ± 5.3	111.7 ± 0.9	(*P ≤ 0.05*)
PQE extracellular 25 ug/ml	6.7 ± 0.2	6.5 ± 8.2	104.2 ± 1.6	100.6 ± 0.4	(*P ≤ 0.05*)
PQE extracellular 50 ug/ml	12.3 ± 0.6	16.4 ± 5	115.9 ± 3.4	107.4 ± 0.7	(*P ≤ 0.05*)
PQE extracellular 100 ug/ml	19.4 ± 0.3	20.5 ± 0.5	126.3 ± 2.0	109.6 ± 0.4	(*P ≤ 0.05*)
PQE extracellular 200 ug/ml	21.2 ± 1.5	43.3 ± 0.3	132.7 ± 2.7	113.5 ± 0.7	(*P ≤ 0.05*)

DISCUSSION

The HAS113 fragments obtained *Streptococcus equi* via the shotgun cloning technique using pUC18 was successfully subcloned into pQE expression system [16, 17]. Huang and Meng, [16] study has proven that the clone was stably maintained after culturing for more than fifteen generations[18]. In this study, plasmid stability testing was conducted, and the result shown that plasmid was well maintained and was very stable in the cell, even after storage on slant agar for one year. The electrophoresis result showed the occurrence of the recombinant plasmid size of 4.4 kb similar to the parental HAS113 clone. The plasmid sequencing result also indicated that plasmid was similar to the parental clone, without any mutation occurred.

The cytotoxicity test of the extracted pQE-HAS113 intracellular and extracellular, were carried out on both breast and prostate cancer cells. The result showed that yellow MTT was reduced to purple when introduced to both cancer cells after the addition of the extracts formazan. Because this reduction occurs only when mitochondrial reductase enzymes are functioning, and therefore conversion is proportional to the quantity of live cells [19]. The effectiveness of an agent in triggering cell death can be determined by comparing the quantity of purple formazan generated by cured cells to the quantity of formazan generated by raw control cells by creating a dose-response curve. Tetrazolium ring is cut by using mitochondrial dehydrogenases in sustainable cells, generating purple MTT formazan crystals that are hydrophobic in nature. The crystals can be melted in acidified isopropanol [20].

Inhibition proportion was estimated from the obtained data collected of the absorbance in order to investigate among the extracts that would be able to slow down or inhibit the growth for both cancer cells. The harvested intracellular and extracellular portions of pQE-HAS113 were tested at different concentrations. The results indicated that the percentage proliferation of both the prostate cancer and breast cancer cells were 2.1% and 7.2% respectively against the intracellular portions of pQE-HAS113. While in case of the extracellular portions of pQE-HAS113 the proliferation rate of both the prostate cancer and breast cancer cells were 6.5% and 6.7% respectively 25 ug/ml concentration. This result indicates that the pQE-HAS113 intracellular extract have the most potent extract to cause toxic to both cancer cells compared to the intra-cellular extract.

This assay also has been used by Carmichael, where the assay was performed on thirty cancerous cell lines of human lungs[21]. In the same year, Romijn reportedly investigated the effectiveness of the MTT assay for quantifying development

modulating impact on cultured cancer cell lines of prostate (PC-3, PC-93, and LNCaP), finding associations between the outcomes attained by MTT-test and those obtained by thymidine integration assay or through DNA measurements [22].

This staining assay was also used by other researchers in their study on the inherent anti-carcinogenic impact of Piper sarmentosum ethanolic extract on hepatoma cell line of human, the same method was applied with 10, 12 and 14 mg/ml of ethanolic extracts, which instigated apoptotic morphological deviations in HepG2 cells [20, 23]. Ribble et al.,[24] compared their method to the typical EB/AO method for detecting apoptosis in Jurkat suspension cell lines and A375 adherent cell lines in normal development and apoptosis-stimulating circumstances. They discovered that their latest EB/AO method yielded results for quantification in both suspension as well as adherent cells that were like those obtained using the conventional EB/AO process. Their strategy cuts the time it takes to complete the test in half, reduces loss to adhering cells, and lessens the chance of dropping floating cells.

There were some researchers conducted previously, which related the effect hya-luronic acid for anticancer properties. One of the studies by Arpicco [25] proposed the conjugation of HA to particulate systems, which could offer chances to focus on can-cerous cells with medication that cannot simply be associated to HA directly [25]. One of the commercial drugs, HA-PTX, also recognized as ONCOFID-P, is currently being tested in phase II trials for ovarian cancer, as well as stomach cancers [25]. HA-But therapy inhibited initial tumour development and lung metastases development from murine Lewis lung carcinoma (LL3) as well as hepatic metastases creation from intra-splenic implantation of LL3 or B16-F10 murine melanoma cells, according to another in vivo investigation [25].

CONCLUSIONS

In this study, we have determined that the recombinant clone, pQE-HAS113 was very stable, even after cultured for more than 15 generations. We have harvested both the extracellular and intracellular fractions of pQE-HAS113 culture to test for anti-proliferative activity on breast and prostate cancer cells. The extracellular fraction, at a concentration of 22.03µg/ml has shown a high percentage of inhibition, 50% to-wards prostate cancer cell. The pQE-HAS113 intracellular extracts have also shown immunomodulatory consequences by enhancing the appearance of inflammatory cytokines. The data indicated that the extracellular faction of pQE-HAS113 culture has a potential to be further develop into anti-proliferative drug. In the meantime, more analysis needs, such as proteomic analysis and toxicity assays need to be carried out to determine the potential of the fraction.

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

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Conflicts of Interest: The authors declare no conflict of interest.

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