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THE EXPRESSION PROFILING OF ALDH3A1, CD44, CD133 AND TNFRSF9 IN HUMAN BREAST CANCER STEM CELLS

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Abstract

Real-time PCR has been used to quantify the gene expression of selected genes to compare among different isolated cancer stem cells. These genes were detected are linked with the reproduction, cell apoptosis and chemotherapy. These genes used as markers to investigate cancer stem cells and as target therapy. In addition, to identify the expression pathways of some genes which related with BCSCs that identified to contribute to tumourgenesis as well as drugs resistance. The expression of these genes will be used to isolate BCSCs from tumour samples or breast cell lines which based on CD44+/CD24-/low, which considers as a crucial marker or ALDH+ phenotypes. Because this isolation will help to analyse the molecular mechanisms which present by self-renewed and differentiation of cells. There is evidence proposed that the isolation of BCSCs will allow the discovery of target therapy and this will remove the mass tumour and breast cancer by using these genes which will up-regulate or down-regulated as it was conducted by several papers.

. In addition, to clarify the expression of selected genes markers in the isolated BCSCs and the tumourgenicity that is a phenomenon degenerated by a subpopulation of the tumour cell which known as cancer stem cells. In this investigation that aimed to isolate BCSCs from the bulk of breast cancer cell. This extraction of breast cancer stem cells was identified by using sphere formation assay. The total RNA was extracted from the isolated breast cancer stem cell in order to detect the expression of novel markers such as;(ALDH3A1) Aldehyde dehydrogenase 3family member A1, the main cancer stem

markers CD44,CD133 and finally tumour necrosis factor receptor super

family 9(TNFRSF9) by run Real-Time PCR.

In this part of our project, we will look at different genes and their expression profiles that are related to the same cells. These isolated stem cells could be tested to quantify the expression profiles of selected genes CD44, CD133, ALDH3A1 and TNFRSF9 as crucial biomarkers.

We investigate cancer stem cells by molecular analysis that includes detection and clarification of the down and up-regulated genes between the strengthened cancer stem cells (spheroid), single and parental cells. Therefore, this isolation allows us to identify the bulk cells to select more specific target therapy.

Introuction

THE ORIGNAL OF BCSCS MARKERS PHENOTYPE

1. ALDH3A1

One of the important markers that play a crucial target in drug process and Oxidation of Aldehydes (Lim *et al*., 2009; Clevers,2011). It has been estimated that this gene found on chromosome 17 in human and involve in cell proliferation. Several studies proposed that ALDH3A1 is expressed in breast cancer tissue and sphere formation assay for MCF7 cells. ALDH3A1 high expression leads to a considerable increase in drug resistance (Muzio *et al*., 2012; Velasco-Velazquez *et al*.,2012). The activity of this gene could be increased due to the expression of metastasis gene Meta dherin (MTDH). The inhibition of ALDH3A1 leads to stopping reproduction of cancer cells (Lorico and Rappa, 2010; Hofving,2012). Studies found that the activity of ALDH can be applied to enhance BCSCs and normal stem cells. ALDH+ cells show heterogeneity similar to the original cells (parental cells) in size. It is reported that CD44+ with ALDH+ phenotypes to improve tumourigenic activity compared with other cells that generate a tumour. Also, both of these markers (CD44+, ALDH+) have been used to investigate breast cancer stem cells by increasing the tumourgenicity. As well as, these markers share with

normal mammary stem cells the capacity to produce mammospheres (Macro A.V. *et al*., 2011; Hermann *et al*.,2010).

.2. CD44

This fundamental marker in BCSCs, which found on chromosome 11on the surface. CD44 is class I transmembrane glycoprotein that has specific receptor from hyaluronic acid (Jaggupilli and Elkord, 2012; Sheridan *et al*., 2006). Several studies show that the main functions of CD44 include ligand binding to the receptor, resistance to apoptosis and marker for prognosis (Li *et al*., 2012; Louderbough and Schroeder, 2000). AL-Hajj *et al* firstly detected the origin of CD44+, in breast carcinoma. Additional studies reported that the presence of CD44, particularly in MCF7 cell line. These cells includeCD44 highly enriched to produce tumour and share the same features for normal stem cells (Wei *et al*., 2012). CD44 found as isoforms help in adhesion interactions. A new study identify that the overexpression of CD44 with another markers is correlated to the investigation of CSCs in several classes of cancer (Dick, 2003; Velazquez *et al*., 2012; Meacham and Morrison, 2013). It has been estimated that CD44+ responsible for several factors of metastatic disease such as movement, drug resistance, proliferation and cell infestation and initiation (Jaggupilli and Elkord, 2012). According to Joensaa *et al*., 60% of tumours was included 90% positive expression of CD44 and the other group were low differentiated (Louderbough and Schroeder, 2011). To investigate the correlation between the CD44 gene-phenotype and proinvasive gene expression QRT-PCR analysis need to be applied (Sheridan *et al*., 2006). Recently, research has shown that CD44 is expressed on progenitor-like cells (Fillmore and Kuper Wasser, 2007).

3. CD133

This gene could be found on chromosome 4 and called prominin 1. It was discovered for the first time in 1997 on the normal stem cells (Labarge and Bissell, 2008; Neuzil *et al*., 2007). Also, CD133 located on the cell membrane and in transmembrane glycoproteins. This protein has been found for a few years as a marker in colon cancer (Labarge and Bissell, 2008). Compared with other CSC markers, CD133 represent the most restricted in the expression. Also, to identify CD133+ subpopulation in breast colon, several studies found it expressed in breast cancer and used as target therapy. Furthermore, CD133 has the capacity to initiate tumour in vivo. The

linkage between this marker and other group allow scientists to study CSCs in different cases especially, in the heterogeneity of CSCs (Florek, Haase *et al*., 2005).

4. TNFRSF9

This gene found on chromosome 1 and forms one of the crucial member of tumour necrosis family which allows the cells to proliferate and can be found in tumorigenesis (Vlad *et al*., 2011). TNFRSF9 plays a crucial role in immune functions and cancer biology (Eckstrum and Bany, 2011).

Material and methods

Materials and reagents for RNA extraction

Equipment and reagents	Suppliers
RNeasy Mini Kit	Qiagan, UK
RNase-Free Water 15-30	Qiagan, UK
Ethanol 600	VWR, UK
X1000 Centrifuge Biofuge	Thermo Scientific, UK
-80 C Freezer	Sanyo 08076722
-20C Freezer	Les 2c0000062
Bench top vortex mixers	Scientific Industries 50HZ
Class II microbiological safety	Envait CI4299
Class II microbiological safety	Walker CI4296
Micro-centrifuge	Heraeus 50/60HZ

Nano Drop 2000/2000c sample	Thermo scientific ,
Spectrophotometer	Labtech 0388
Qpcr Machine	R Corbett R070571
Red sterile tube25ml	SARSTEDT, Germany 62.547.254
Yellow sterile tube 25ml	SARSTEDT

TABLE 3.3 Materials and tools for RNA extraction

Material and reagents for cDNA synthesis

Material and reagents	Suppliers Catalog number
Total RNA 5µg Oligo (dT)18 1µl 10mM dNTP 1µl 5x RT Buffer 4ml RiboSafe RNase Inhibitor 1ml Tetro Reverse Transcriptase(200m/ml) DEPC treated water Made up to 20 µl	
Tetro cDNA synthesis kit	Bioline, UK. 30 reaction BIO-65042
0.5 ml Micro Tubes	Sarstedt 72.699
1.5 ml Micro Tubes	Sarstedt 72.690.001
15 ml Tubes	Sarstedt 62.554.502
2ml Tubes	Sarstedt

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	72.695
50ml Tubes	Sarstedt 62.547.254
Pipette tips 1000 ml, blue	Sarstedt 70.762
Pipette tips, yellow	Sarstedt 70.760.002

Table 3.4 material and tools for c DNA synthesis.

RNA EXTRACTION

The total RNA was extracted from MCF-7 cell lines by using an RNeasy mini kit (Qiagan, UK) for each type of cells separately; parental cells and single cells that were isolated from sphere cells in one flask, in order to investigate the expression profile of the genes of interest. By following the manufactures protocol, the cells were washed with PBS twice and centrifuged for 5 minutes at 1500 rpm. The cells were homogenised by lysis for 30 seconds to break down the cell membrane. After the cells had been homogenised, 600µl RLT plus Buffer and six µlB-mercapto-ethanol were added. The contents were transferred with pipette lysate into a QIA shredder then centrifuged for 3 minutes at 15,000 rpm. The supernatant was collected and the column was discarded. The centrifugation was down for 30 seconds at 10,000 rpm. 600µl of 70% ethanol was added to the tube and 700µl of the sample was transferred to RNeasy spin column twice. This column was centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded and the column retained. 700µl of RW1 Buffer was added and centrifuged for 15 seconds at 10,000 rpm. The flow was discarded and the column was maintained. 500µl of RPE Buffer was added and centrifuged for 15 seconds at 10,000 rpm. The flow was removed; this step was repeated two times. The centrifugation was for 2 minutes at 10,000 rpm the second time. The flow was discarded and this time the column was replaced with a new collection tube. The new tube was centrifuged for 2 minutes at 13,000 rpm. The column was retained and the flow removed and the column was replaced with the new collection tube. For the new tube, 15-30 µl RNase-free water

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was added and centrifuged for 1 minute at 10,000 rpm. Finally, the column was discarded at this step and the flow was retained. The total isolation RNA was collected in a small new tube and stored at-80^{o}C. The result of RNA was quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific, UK). This experiment was replicated three times for parental, single and sphere cells.

REVERSE TRANSCRIPTION OF TOTAL RNA (cDNA)

The total isolation of RNA samples that is been converted to cDNA synthesis by using a Tetro c DNA Synthesis Kit (Bioline, UK). 5µg of RNA was used and the solution was vortexed and centrifuged briefly. The priming mix was prepared on ice in RNase reaction tubes. The mix included 5mg RNA, 1µ Oligo (dt) 18, 1ml of 10mM Dntp mix, 4ml of 5x RT Buffer, 1ml of Ribo safe RNase inhibitor and 1ml Tetro reverse transcriptase (200m/ml), and finally up to 20 DEPC-treated water was added to the mix. The latter was mixed gently by pipetting and incubating at 45c for 30min. The incubation was repeated by increasing the temperature to 85C for 5 minutes. The samples were stored at -20C for long term storage and some of these samples were used directly for the Q- PCR experiment.

PRIMERS DESIGN

The reverse transcription of RNA was applied to identify the expression profiles of the ALDH3A1, CD44, CD133 and TNFRS9 genes and the housekeeping gene (GAPDH). The mRNA sequences detailed were obtained from the (NCBI) website National centre for biotechnology. The primers for our genes have been achieved from the same website and used in Q-real time PCR.

Primers	The Gene Sequences (5'-3')	Melting temperature
ALDH3A1	FP tcatcaaccagcgtgagaag RP aggtgatgtggacgatgaca	57.3
CD44	FP actctgctgcgttgtcattg RP gtggaagatttggacagga	57.3

CD133	FP tggcgttgtactctgtcagg RP tccaacagggctatcaatcc	59.4
TNFRSF9	FP gccccgtttaaacaacagaga RP tccgcagatcatctccttc	57.3
GAPDH(Housekeeping gene)	FP cgaccactttgtcaagctca RP ccctgttgctgtagccaaat	57.3

Table 3.5 The Selected Genes for q-PCR

THE REAL-TIME PCR (Q-PCR)

The expression profiles of our genes of interest which were obtained after c DNA synthesis that was converted from different stages in various types of cells in real time PCR. The real-time PCR was applied by using Rotor-Gene 6000 (Corbett Research, UK) machine with 27 wells size. The master mix was prepared by applying the following reagents:

Reagents for Master Mix	***The Volume for One Reaction***
RNase-free water	*4.25 (µl)*
Forward primer	*0.5 (µl)*
Reverse primer	*0.5 (µl)*
SYBR Green	*6.75 (µl)*
water for control	*0.5 (µl)*
c DNA for samples	*1 (µl)*

Table 3.5 Using Reagents for real-time PCR.

This experiment was carried out under highly hygienic conditions (in the hood). The tubes were prepared as followed:

Two parental cells, two single cells, two spheres (in normal media + normal culture flasks) and spheres in new media with new culture flasks. This experiment was done for each gene separately and then for all the genes in one run. The master mix which was classified among 20 tubes at volumes 12.5µl and 1µl were added from the samples to each tube and 0.5 µl RNase-free water was added to the blank tube for the control. Each of the c DNA samples which was applied to different genes and the housekeeping gene as well for a controlled reaction.

setting the q-PCR program by using the Corbett machine

The program was opened and set up on the '3-steps with melt' by selecting a new run. The 72 well rotor (blue) needs to be chosen because it corresponds to the universal tubes. The operator information needs to be loaded and checked that the reaction volumes are 12.5µl. The most important feature was the 'Holding' temperature setting which was 95^{o}C for 5 minutes. Cycling needs to be identified as well by selecting the 'Denaturation' of 95^{o}C for 20 seconds. The 'Annealing' temperature was different for each gene depending on the primer as was mentioned in table (…..) (The average for all the primers was taken between 57.3 and 59.4, which was 57.72^{o}C). Finally, the 'Extension' was 72^{o}C for 20 seconds. Then cycling was repeated 40 times. The run can be started after all the steps were checked carefully, and the samples were edited with each specific name on it. In the end, the programme should be saved so that the data can be analysed.

STATISTICAL ANALYSIS

The mammospheres assay results were shown under the microscope by taking photos for different types of spheres in different days and using two different methods; normal condition and non-adherent culture. The q-pcr data was analysed by using Excel sheet software that is presented as the mean threshold cycle (CT) value calculated for analysis of the housekeeping gene (GAPDH) which was used as a control gene. This helps to identify the values of the genes of interest. This involved identification of CT1 and CT2 values for the replicated samples and taking the average of them. Another method was related to investigating the housekeeping gene values (GAPDH) which includes detecting the values from the results by selecting 'Replicate

take off' value that is related to the samples that are used in the reactions. The average was done for all the concentrations. Then, by using an Excel sheet to calculate all samples based on the value of the Housekeeping gene. The final results will be calculated and the mean for each sample was gained by using a Graph pad prism and the Error Bar was obtained. A one way ANOVA analysis was applied to optimize the significance among the gene expression for different MCF7 cells *P value<0.05 and **P value <0.01. Results from all markers were obtained in duplicate.

Results

REAL-TIME PCR RESULTS

Expression profiles of selected genes in parental, single and sphere MCF7 cells formed from different culturing (normal and non-adherent conditions) by using q- PCR.

1 THE EXPRESSION PROFILE OF ALDH3A1

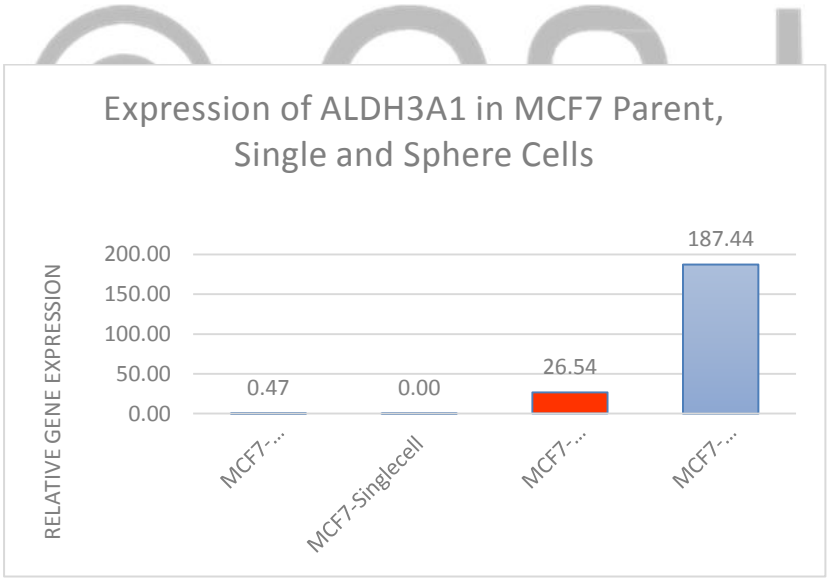

Figure 4.3 Gene Expression Profiles of MCF7 Parental, Single and Spheres cells in both Culture Conditions.

In this assay, the RNA was isolated from MCF7 parental, single and spheres by using a Qiagan Kit. The whole RNA was extracted and converted to c DNA, which was used finally in the q-PCR reactions. The real-time PCR was applied in order to quantify the results by using the housekeeping gene (GAPDH). The final reaction of Q-PCR results clarify that the ALDH3A1

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gene shows up-regulation in MCF7 spheres in the new conditions culture and new media compared to parental, single and spheres MCF7 in normal conditions cells which were showing no expression for ALDH3A1.

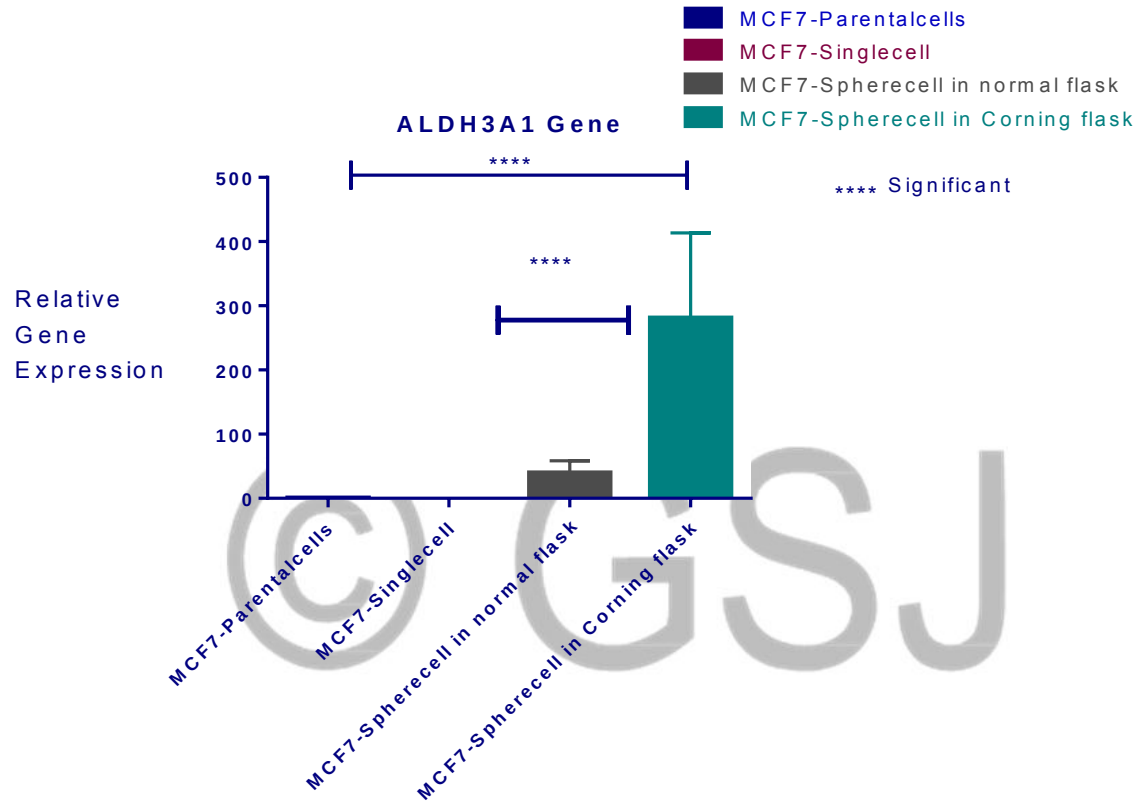

Figure 4.4 ALDH3A1 Expression

Figure 4.6 shows the statistical analysis by using Graph-pad prism 6. The up-regulation of ALDH3A1 was significantly proven for spheres in corning flask (non-adherent conditions and new media) compared with spheres in normal conditions, single and parental. The mean+ - shows the P values are <0.0001 and the ANOVA one way test for multiple comparison show significant results **** for MCF7 sphere cells in non-adherent conditions. The data show the mean value from triplicate experiments. This was measured by One way ANOVA ****P= <0.0001. The multiple comparison show significant result **** for MCF7 spheres in non-adherent conditions.

This e**xperiment was replicated three times. (See Appendix3)**

2 THE EXPRESSION PROFILES OF CD44

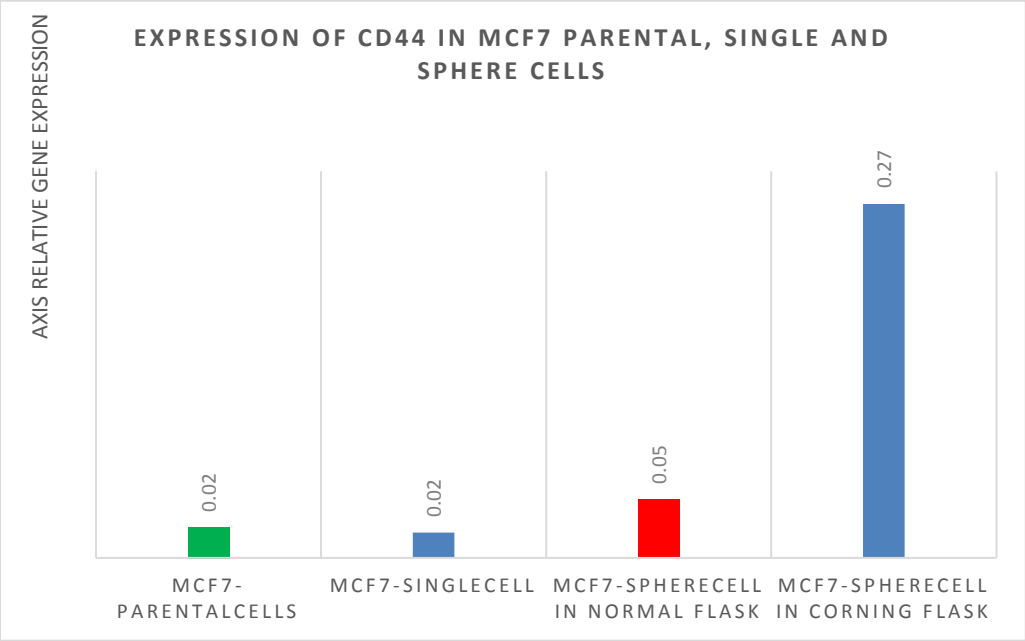

Figure 4.5 Gene Expression Profiles for MCF7 parental, single and spheres in both culturing assays.

In this assay, RNA was isolated from fresh MCF7 parental, single and spheres cells by using a Qiagan Kit. The isolated RNA was converted directly to c DNA synthesis, which was used finally in the q-PCR reactions. The real-time PCR was applied in order to quantify the results by using a housekeeping gene (GAPDH). The final reaction of Q-PCR results showed up regulation of the CD44 gene in MCF7spheres (which were produced from a new flask) compared to the MCF7 parental cells. This experiment was repeated three times. (See Appendix page3).

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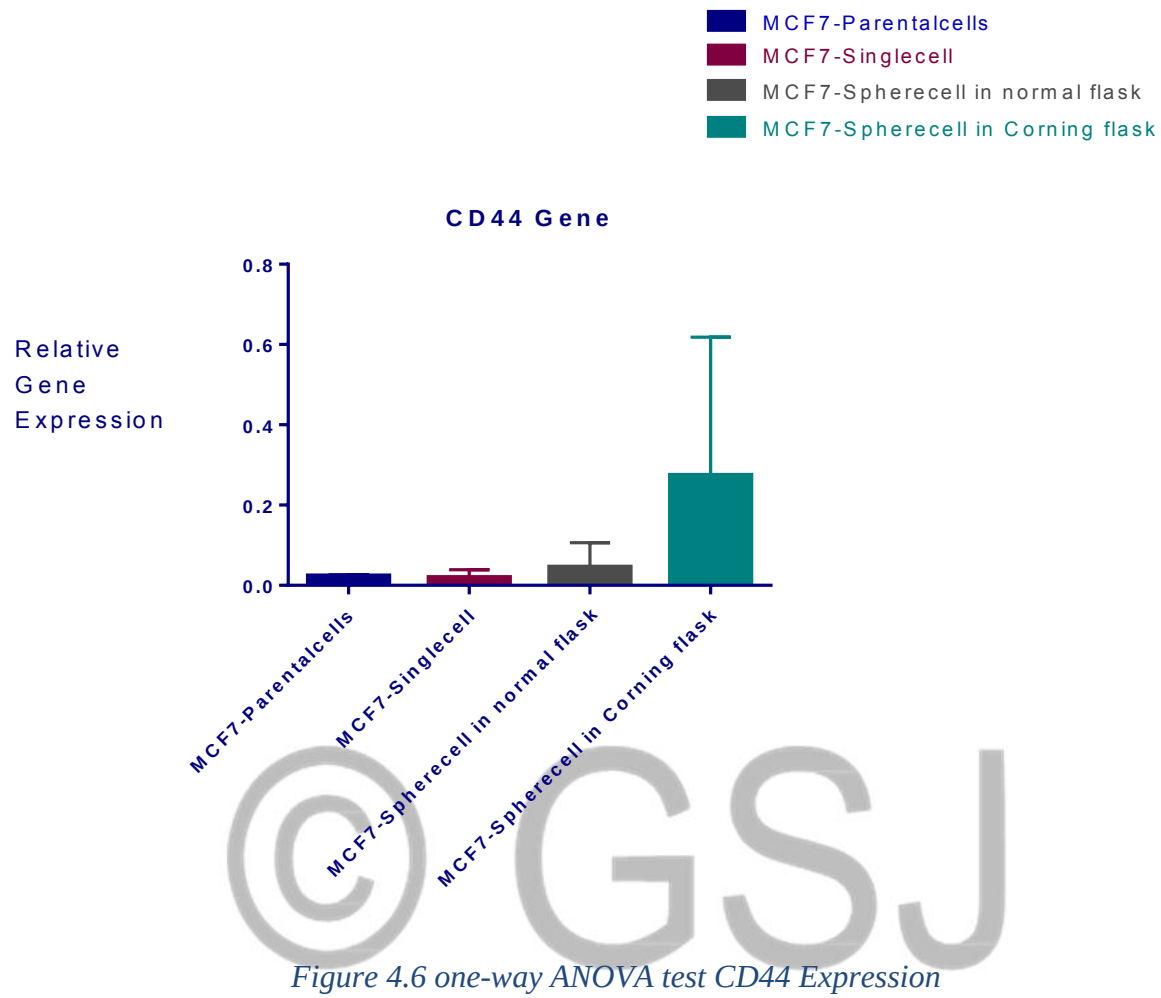

Figure 4.6 one-way ANOVA test CD44 Expression

Figure 4.8 shows the statistical analysis by using Graph-pad prism 6. The up-regulation of CD44 was noticed in sphere cells which were in corning flask (non-adherent conditions and new media) compared with spheres in normal conditions, single and parental. However, the result was not significant. The mean+ - for the ANOVA one way test P value =0.4755. This experiment was done in duplicated (See Appendix3).

There was considerable up-regulation in MCF7 spheres cells in non-adherent condition. That indicates there were significant differences in the expression of CD44 amongst parental, single and sphere MCF7 cells, in spite of that was not approved statistically.

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3 THE EXPRESSION PROFILES OF CD133

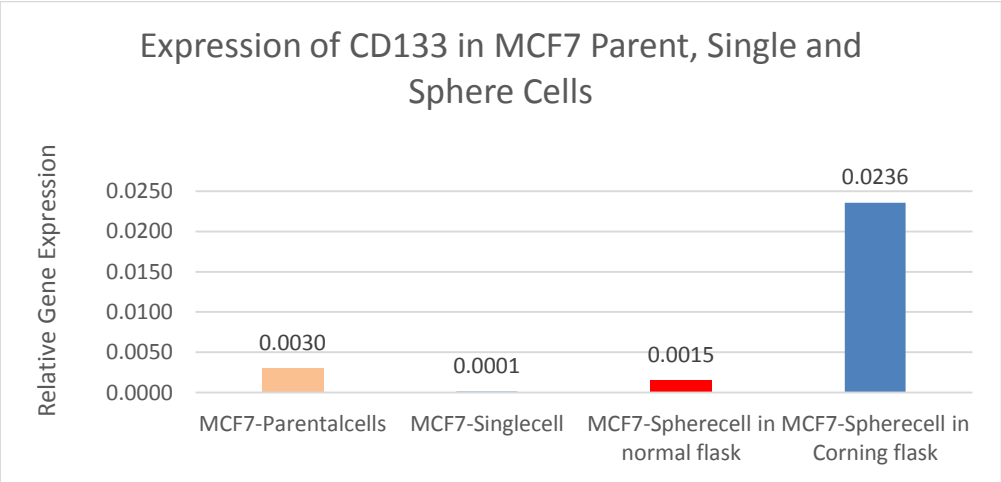

Figure 4.7 Expression profiles of MCF7 parental, single and spheres cells (in both conditions).

In this assay, RNA was isolated from fresh MCF7 parental, single and spheres cells by using a Qiagan Kit. The isolated RNA was converted directly to c DNA synthesis, which was used finally in the q-PCR reactions. The real-time PCR was applied in order to quantify the results by using a housekeeping gene (GAPDH). The final reaction of Q-PCR results shows up-regulation of CD133 gene in MCF7spheres (which were produced from a new flask) compared with MCF7 parental cells.

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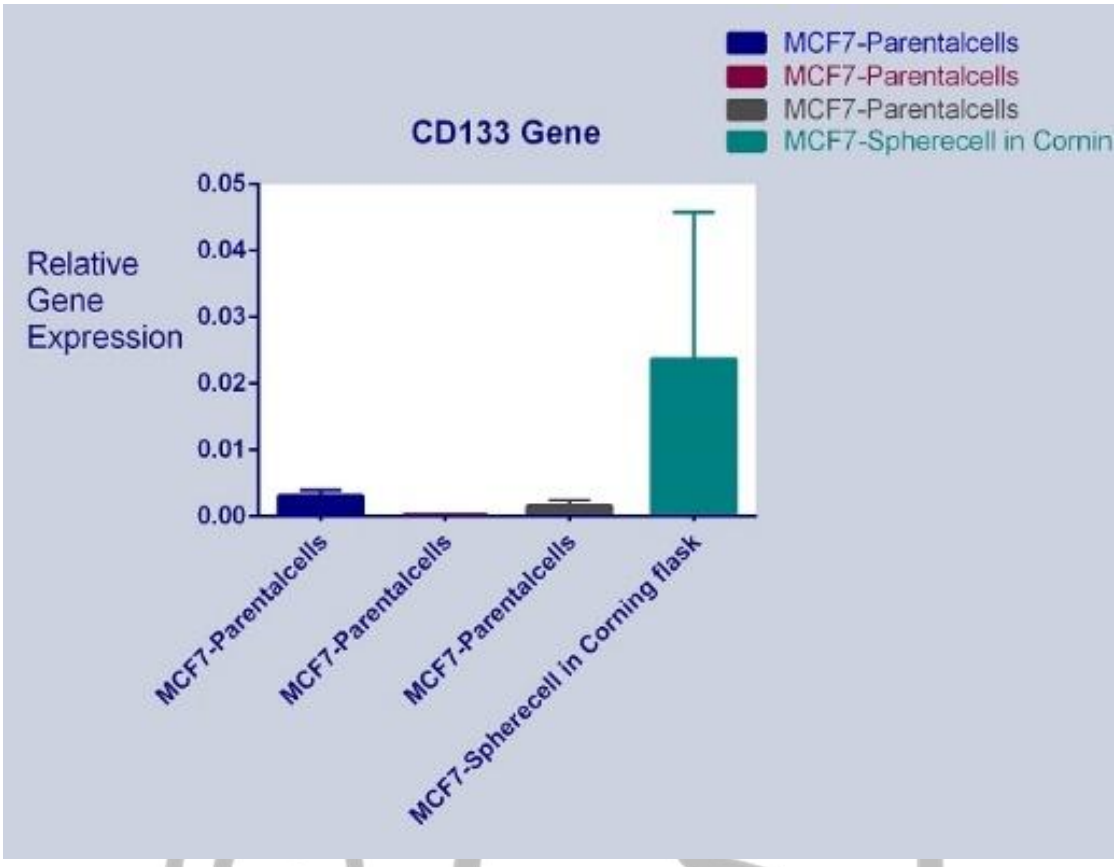

Figure 4.8 one-way ANOVA test CD133

Figure 4.10 shows statistical result by using one-way ANOVA values which demonstrate the noticeable up-regulation of CD133 gene in MCF7 spheres cells which had been cultured in the non-adherent condition in a corning flask. This data was repeated twice. However, the data was not significant statistically, the P value= 0.3930.

4 THE EXPRESSION PROFILES OF TNFRSF9

In this assay, RNA was isolated from fresh MCF7 parental, single and spheres cells by using (Qiagen, Kit). The isolated RNA was converted directly to c DNA synthesis, which was used finally in the q-PCR reactions.

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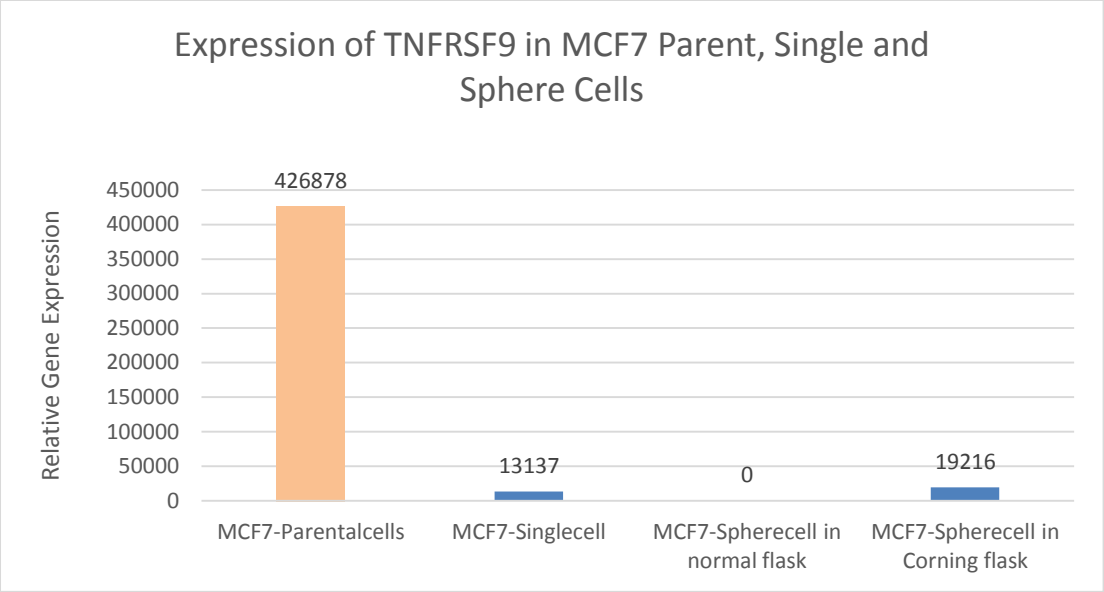

Figure 4.9 gene expression profiles of MCF7 parental, single and spheres cells in The real-time PCR

Figure 4.11 shows the gene expression profiles of MCF7 parental, single and spheres cells (in The real-time PCR was applied in order to quantify the results by using a housekeeping gene (GAPDH). The final reaction of Q-PCR results shows downregulation of TNFRSF9 gene in MCF7spheres (which produce from a new flask), spheres (which were produced in normal flask and media) and single compared with MCF7 parental cells in different methods of producing spheres).

Discussion

. This part of the investigation was to detect and identify the expression of different selected genes as biomarkers which forms an ideal methods to help in early diagnosis of breast cancer (Gromov *et al*., 2010; Yang et al.,2009). Shawn and his colleagues clarified that MCF7 cells can be generated for more than twenty passages (Shwan *et al*., 2012). Recently, a variety of researchers have studied the correlation between cancer stem cells and the features of self-renewal, differentiation and proliferation (Yi *et al*., 2013; Chaves, Garimella and Lipkowize,2010).

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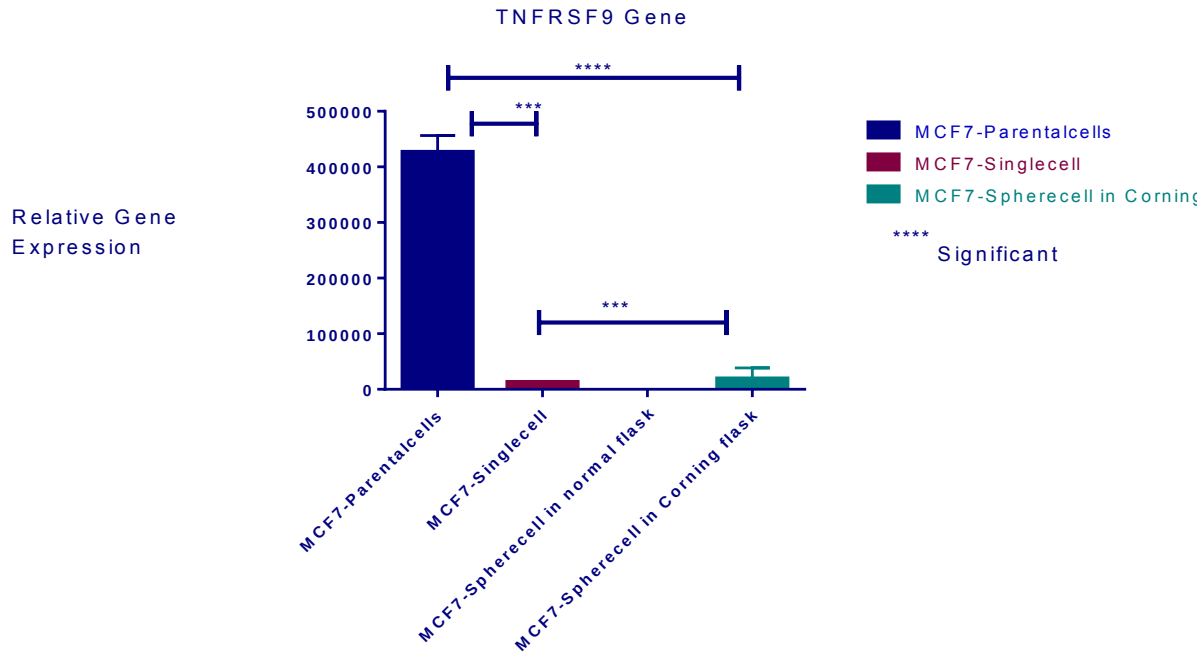

Figure 4.10 one-way ANOVA test TNFRSF9

Figure 4.12 illustrates that the standard deviation values of the gene expression of TNFRSF9 were up-regulated in MCF7 parental cells compared with less upregulation of MCF7 sphere cells. The data show significant result the ****P value <0.0001 and the multiple comparison for all the type of cells show P***, P*** and p*** for the parental, single and spheres in non-adherent condition. This result shows the statistically significant difference in the expression of TNFRSF9 compared with other MCF7 cells.

Pham *et al*., pointed out that mammospheres assay presents as a method with high limitations because this method might include a small population of cells that cannot exhibit CD44 phenotypes. Therefore, they found that BCSCs, which had been isolated from mammoshere formation present as less pure and had a lesser resistance to drugs (Pham *et al*., 2012). This finding has the approval by several researchers such as Morel *et al*. It demonstrates that tumorigenic CD44+ cells can generate from human mammary epithelial cells. After isolation of mammospheres in different ways from MCF7 cells, the gene expression was determined in

parental, single and sphere MCF7 cells (in both culture conditions). This was done by performing real-time PCR.

In a study carried out by Liu et al., (2015) they were able to isolate side population (SP) from brain tumour by using spheres formation assay. Their finding illustrates that SP cells had the same features of CSCs. Also, the expression of stem cells markers was increased which was consequently increased and that refer to increasing in drug resistance, invasion and recurrence of tumour. Therefore, for these reasons cells were highly and quickly able to self-renewal and regenerate mammospheres.

Gene Expression profiles by using Q- real time Pcr results:

Real-time PCR was carried out in order to determine the expression of a housekeeping gene and the selected genes as markers for CSCs. In the present study, c DNA expression profiling of human breast cancer is widely used to identify tumour cells depending on the gene expression (Chavez *et al*., 2010; Bieche et al.,1998). A variety of studies have focused on human cancer cell lines because they play important roles in studying and understanding the molecular aspects, therapy and the biology of cancer in different types of malignancies (Chavez *et al*.,2010). We validated the real-time PCR method developed for the quantification of gene expression in isolated cancer stem cells. This technique is based on real-time PCR analysis of gene amplification. The main motive for using this analysis was to gain comparable PCR results and to know the quantity of gene expression. Using small tubes helped to avoid contamination of the samples. Another reason is this method gives fast and simple results. In addition, the calculation of Ct values give very specific and valid results (Bieche and Olivi, 1998). Spheres cells were isolated from MCF7 cell lines and the gene were observed in all selected genes were shown upregulated to the spheres in non- adherent conditions with exception of TNFRSF9 gene which shows down regulated [Figure 4.5,6,7,8,9,10,11 and 12].

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ALDH3A1 EXPRESION

In this study, the expression of ALDH3A1 was upregulated in MCF7 spheres (Corning, US) and compared with spheres in a normal flask, and parental and single cells (figure). The increase in the expression of this gene was proven statistically significant ****P. The high expression of ALDH3A1 refers to mammospheres that were formed from MCF7 cells. They are able to self-renew and differentiate when the cells grow in normal and non-adherent conditions but this happens at different rates. Therefore, it is possible that these cells can generate BCSCs (Muzio *et al*., 2012).

CD44 EXPRESION

In this experiment, CD44 expression shows an upregulation in MCF7 sphere cells compared with parental, single and sphere cells in normal conditions. However, statistical analysis shows an insignificant result.

AL-Hajj and his colleagues found that BCSCs were heterogeneous. Also, the cell markers were expressed highly with CD44. This gene is considered as an adhesion molecule. For the first time, in the findings by AL-Hajj and co-workers, BCSCs had been identified as a heterogeneous population that are also a phenotypic tumorigenic population. A study conducted by Wang *et al*., found that after 3 weeks of culturing breast cancer stem cells in mammospheres system, there was a considerable increase in CD44++ cells. Therefore, they were able to prove that BCSCs can be generated from MCF7 cell lines in non-adherent flasks to form spheroids. Compared with parental and single cells, MCF7 cells were loose and floating in the media (Wang *et al*., 2014). These results agree with the results in our project. A further experiment in vitro used mammospheres assay indicating that MCF7 cells have the ability to self-renew mammospheres. After four weeks of culturing they found that MCF7 breast cancer cell lines classified as CD44+ non-pervade cells are able to invade cells in both pathways (Owens and Naylor, 2013). More recent experimentation was done by Abboodi, 2014 to illustrate that the expression of CD44 is higher in cancer stem cells compared to MCA MB cells when cultured in different conditions.

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This finding was similar to our work as we can see in (Figure); the CD44 shows a high expression in MCF7 sphere cells produced in new conditions (Abboodi, 2014).

CD133 EXPRESION

In this investigation, CD133 expression was upregulated in MCF7 sphered (Corning, US) and compared with parental, single and sphere (normal flask) MCF7 cells. This result was statistically insignificant. The expression of CD133 in single cells and parental cells was at the same level.

Another study carried out by Lorico and Rappa, identified the expression of CD133 in breast cancer stem cell spheres of MARY-X xenograft model of IBC. In this finding, they were able to detect the MARY-X spheroid as CD44+, ALDH3A1 and CD133+ (Florek, Haase, *et al*., 2005). Researchers assume that increasing the expression of marker genes in the surface link to the stem cells phenotype and cell lines. This finding is associated with culturing spheres and leads to an increase in tumourgenicity at the end.

Furthermore, a study conducted by Saadin and White, illustrated the importance of other markers such as; cells with high ALDH had a large formation of spheres whilst when they had low ALDH there was no formation of spheroids. This finding means that ALDH forms a crucial marker in cancer stem cells. Similar results were obtained for CD133 as a marker in Non-small lung cancer (Saadin and White, 2013). The further research used the expression of aldehyde dehydrogenase assay to isolate CSCs population in different cell lines. In order to give strong evidence for the existence of cancer stem cell population in BCSCs lines, aldehyde dehydrogenase (ALDH) has been used in this study. By using the activity of ALDH, researchers were able to isolate cancer stem cells. In addition, CD44 and CD133 were used as well as surface markers (Jauffret *et al*., 2009).

TNFRSF9 EXPRESION

The expression of TNFRSF9 was downregulated in MCF7 Spheres cells compared with parental cells (FIGURE). However, this result was highly significant statistically. It is estimated that this

gene is essential at the beginning of tumorigenesis and damage to this gene leads to the initiation of a tumour (Li *et al*., 2009).

A study carried out by Patsialou *et al*., shows that TNFRSF9 is downregulated when they analysed the result by using real-time pcr for the isolation of MDA-MB-231 cells (Patsialou *et al*., 2012). These findings agree with our result with the exception of the use of MCF7 cells, which shows the down-regulation of sphere cells in both conditions and up-regulation in parental cells. A variety of literature identifies that down regulation of apoptotic genes in the isolation of CSCs plays a crucial role in the resistance of these cells to drug therapy and radiotherapy. Gene expression was validated by Q- RT-PCR analysis (Jauffret *et al*., 2009; Weigelt, Kreike and Filho, 2009). In their findings, the genes CD44+ and ALDH+ were highly expressed in breast cancer stem cells when they performed new culture spheres. Therefore, this finding considers it is crucial to understand breast cancer drug resistance and validated target therapy (Abboodi, 2014).

In this work, we were able to isolate tumorigenic cancer cells based on different markers expression. We identified cell markers to investigate tumour heterogeneity in various cell populations. The expression of three selected genes ALDH3A1, CD44 and CD133 in our data show up-regulation of MCF7 spheres in non-adherent conditions compared to spheres in normal conditions, single and parental cells as well. In contrast, TNFRSF9 expression shows downregulation of spheres in both conditions and single cells compared with MCF7 parental cells show up-regulation.

There are scores of evidence indicates that cancer stem cells can provide us with an ideal target therapy (chemotherapy and radiotherapy) due to the ability of CSCs to resist the different type of drugs. New methods, such as the use of biomarkers, need to be discovered to isolate CSCs, which plays a crucial role in the clinical diagnosis, particularly in early diagnosis. Therefore, the gene expression provides a vital tool in the prognostic factors of breast cancer.

Summary

By using RT-QPCR, we were able to evaluate quantitatively the expression profile of selective genes; ALDH3A1, CD44, CD133 and TNFRSF9 in breast cancer stem cells which have been isolated from MCF7 cell lines. The results of genes expression profiles were obtained by Q-pcr which represents a very sensitive equipment which allow to the scientists to detect on gene deletion and concrete outcomes in real time for each individual gene in each cycle (Strati *et al*., 2011). As well as, analyse a big numbers of samples in short time, this will be the first step to help in the clinical diagnosis. Therefore, the gene amplification can be used as markers for early diagnosis of cancer and choice of treatment (Bieche and Olivi, 1998). In addition, to provide an ideal therapy for cancer, CSCs have been isolated in order to achieve more efficient treatment for some cancers by focusing on the ideal drugs for each type of tumour cells (Shackleton *et al*., 2009).

FUTURE WORK

The theory of cancer stem cells represents an ideal target therapy. Due to the highly resistance to drugs, the need for further studies to concentrate on the cancer stem cell mechanisms, breast cancer heterogeneity which is believed that it sustains from the presence of BCSCs population and focus more on the biomarkers which play a key roles to investigate the cell population. Further experiments need to be done in order to test the combination of markers and additional markers need to be discovered. Moreover, it is required examine specific gene expression to target BCSCs population of a variety of breast cancer subclasses and understand the signalling pathways in order to clarify cancer prognosis (Lorico and Rappa, 2010). This work was specific for MCF7 breast cancer cell lines. This limitation for only this type of cells and not for the tissues. Also, using qpcr might be very limited compared if microarray or flow cytometry was performed as well to quantify the expression of genes of interest.

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