Study on Breast Cancer Stem Cells and their Role in Anti-Cancer Drug Response

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CERTIFICATE

This is to certify that the thesis entitled **Study on Breast Cancer Stem Cells and their Role in Anti-Cancer Drug Response** and submitted by **SANTHI LATHA PANDRANGI** ID No.: **2009PHXF010P** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

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"Somewhere, something incredible is waiting to be known."

....**Carl Sagan**

सर्वतीर्थमयी माता सर्वदेवमयः पिता|
मातरं पितरं तस्मात् सर्वयत्नेन पूजयेत्||

(Mother is the embodiment of all pilgrimages, father is the embodiment of all deities. Hence, mother and father are to be revered with all the efforts)

Dedicated to my parents

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- Santhi Latha Pandrangi

Abstract

Breast cancer constitutes the most common malignancy and the most common cause of cancer related deaths in Indian women. Incidence of breast cancer in India is rising steadily. In India, the average incidence rate for breast cancer is 16.5 per 100,000, varying from 22-28 per 100,000 in urban areas to 8 per 100,000 in rural areas. Also a significant proportion of Indian breast cancer patients are younger than 35 years of age. In India, the etiology of breast cancer appears to be different from that in the west, with early onset, short disease-free interval and high mortality. But there is no breast cancer cell line available from Indian population in the international or national cell culture repository. Despite recent therapeutic advances, locoregional and systemic disease recurrence remain an ever-present threat to the health and well being of breast cancer survivors. Disease recurrence originates from residual treatment resistant cells called the Cancer Stem Cells (CSCs). CSCs are defined as being able to self-renew and are the origin of other cancer cells that contribute to the mass of the tumor. They are responsible for maintaining the tumor and have been hypothesized to lead the invasive front of the tumor and contribute to metastatic seeding. CSCs were first discovered in acute myeloid leukemia and subsequently in solid tumors, including breast, pancreas, colon, glioblastoma, and others. Previous studies demonstrated that their quantification in tumor tissues would have significant prognostic value. Breast cancer stem cells (BCSCs) from tumor tissues/ cell lines can be identified and sorted out based on the presence of markers like $CD44^{+}/CD24^{-}$ or aldehyde dehydrogenase enzyme ($ALDH1A1^{+}$ cells) and can be further enriched and propagated in suspension cultures as 'mammospheres'. The mammosphere system offers an in vitro model to study mammary stem cell biology and pathogenesis. However, little is known about the behavior of these cells in long-term cultures. The inability to maintain cancer stem cells in an undifferentiated state *in vitro* has further marred their characterization.

This thesis is a contribution to the knowledge about the importance of Indian breast cancer cell lines and breast cancer stem cells and how they might interfere with the health. More specifically the aims have been 1) to establish and

characterize breast cancer cell lines from primary tumors of Indian breast cancer patients 2) to isolate and quantitate breast cancer stem cells from breast cancer cell lines and 3) to study the effect of anticancer drugs on breast cancer cell lines.

To achieve the aims we have established two novel triple negative breast cancer cell lines, NIPBC-1 and NIPBC-2 from early onset primary tumors of two breast cancer patients diagnosed with infiltrating duct carcinoma of breast. Both the cell lines were found to be of non-basal origin. Characterization of these cell lines showed non-basal origin with expression of epithelial specific antigen and cytokeratin 18 and presence of microfilaments and secretary vesicles, microvilli, tight junctions and desmosomes on ultra-structural analysis. Both the cell lines showed anchorage independent growth and invasion of matrigel coated membranes. Karyotype analysis showed aneuploidy, deletions and multiple rearrangements in chromosomes 7, 9, X and 11 and Isochromosomes 17q in both the cell lines. P53 mutational analysis revealed no mutation in the coding region in both the cell lines; however NIPBC-2 cell line showed presence of heterozygous Arg/Pro polymorphism and NIPBC-1 showed presence of homozygous Arg/Arg allele at codon 72 of exon 4. Both the cell lines showed presence of CD $44^{+}/24^{-}$ breast cancer stem cells and capability of producing mammospheres on culture. We have also identified and sorted out $ALDH^{+/bright}$ BCSCs from various commercially available breast cancer cell lines and propagated and enriched them in suspension cultures as 'mammospheres'. Studying cancer stem cell behavior is important in understanding cancer pathogenesis. Since extensive self-renewal potential is the hallmark of stem cells, we undertook a detailed functional characterization of human mammospheres over long-term passages. With increasing passages, mammospheres showed a dynamic increase both in the number of MFUs (mammosphere forming units) and sphere forming efficiency until the second passage followed by a dramatic reduction. We have also observed an increase in the number of smaller sized spheres relative to the larger ones over multiple generations of mammospheres.

It is further reported that patients with elevated levels of CSCs would more likely suffer from an aggressive form of disease that is comparatively resistant to currently employed therapeutics. Hence, targeting breast cancer stem cells

(BCSCs) offers a promising strategy for breast cancer treatment. We examined the plant alkaloid ellipticine, for its efficacy to inhibit the expression of ALDH1A1-positive BCSCs in comparison with paclitaxel which is a clinically used drug, by *in vitro* and *in silico* methods. At 3mM concentration, ellipticine decreased the expression of ALDH1A1-positive BCSCs by 62% (p=0.073) in MCF7 cell line and by 53% (p=0.024) in SUM159 cell line compared to vehicle treated cultures. Ellipticine significantly reduced the formation of mammospheres whereas paclitaxel enhanced mammosphere formation in both the treated cell lines. Interestingly, when treated with a combination of ellipticine and paclitaxel the percentage of ALDH1A1-positive BCSCs dropped by several fold *in vitro*. A homology model of Homo sapiens ALDH1A1 was built using the crystal structure of NAD bound sheep liver class I Aldehyde Dehydrogenase [pdb id: 1BXS] as a template. Molecular simulation and docking studies revealed that the amino acids Asn-117 & 121, Glu-249, Cys-302 and Gln-350, present in the active site of human ALDH1A1 played a vital role in interacting with the drug.

Taken together we propose that our two established breast cancer cell lines NIPBC-1 and NIPBC-2 can serve as novel *in vitro* models to study mechanisms underlying breast tumorigenesis in younger age group patients and also identification of new therapeutic modalities targeting cancer stem cells. Also the available evidence suggests that the plant alkaloid ellipticine reduces the proliferation and self renewal ability of ALDH1A1- positive BCSCs and can be used in combination with a cytotoxic drug like paclitaxel for potential targeting of BCSCs.

This thesis describes the above work in detail.

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Abbreviations

AAR	:	Age-adjusted Rate
ABC G2	:	ATP Binding Cassette Gene 2
ADT	:	Autodock tools
AJCC	:	American Joint Committee on Cancer
ALDH	:	Aldehyde Dehydrogenase
ALDH1 A1	:	Aldehyde Dehydrogenase 1 Class A1
AML	:	Acute Myeloid Leukemia
ATM	:	Ataxia-Telangiectasia Mutated
BCRP1	:	Breast Cancer Resistance Protein 1
BCSC	:	Breast Cancer Stem Cell
BLAST	:	Basic Local Alignment Search Tool
BRCA1	:	Breast Cancer 1 Gene
BSA	:	Bovine Serum Albumin
CAMs	:	Cell Adhesion Molecules
CK	:	Cytokeratin
c-myc	:	Myelocytometosis Gene
CPMV	:	Cowpea Mosaic Virus
CSC	:	Cancer Stem Cell
DAB	:	Diamino benzidine Tetrahydrochloride
DCIS	:	Ductal Carcinoma in situ
DEAB	:	Diethyl Amino Benzaldehyde
DMEM	:	Dulbecco's Modified Eagle Medium
DMEM/F12	:	Dulbecco's Modified Eagle Medium Nutrient Mixture F12
DMSO	:	Dimethyl Sulphoxide
dNTP	:	Deoxy Nucleic Acid Triphosphate
ds DNA	:	Double stranded DNA
E-Cadherin	:	Epithelia Cadherin
EDTA	:	Ethylene Diamino Tetra Acetic acid
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermial Growth Factor receptor
EMA	:	Epithelial Membrane Antigen

EpCAM	:	Epithelial Cell Adhesion Molecule
ER	:	Estrogen Receptor
erb B2	:	v-erb-b2 Avian Erythroblastic Leukemia Viral Oncogen Homolog 2
EtBr	:	Ethedium Bromide
FACS	:	Flourescence Activated Cell Sorter
FBS	:	Fetal Bovine Serum
gromacs	:	Groningen Machine for Chemical simulations
GSK3	:	Glycogen Synthase Kinase 3
HBSS	:	Hank's Balanced Salt Solution
HRP	:	Horse Radish Peroxidase
IARC	:	International Agency for Research on Cancer
ICMR	:	Indian Council of Medical Research
IDC	:	Invasive Ductal Carcinoma
IGF2	:	Insulin-like Growth Factor 2
ILC	:	Invasive Lobular Carcinoma
Kb	:	Kilo base
LCIS	:	Lobular Carcinoma in situ
MACS	:	Magenetic Cell Sorter
MD	:	Molecular Dynamics
MDR1	:	Multidrug Resistant 1 Gene
MFU	:	Mammosphere Forming Unit
MTT	:	M3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	:	National Center for Biotechnology Information
NCCP	:	National Cancer Control Program
NCRP	:	National Cancer Registry Project
NOD	:	Non-obese diabetic Mice
NSP	:	Non-side Population
p21	:	21 kda Protein
p53	:	53kda Protein
PBCR	:	Population Based Cancer Registry
PBS	:	Phosphate Buffer Saline

PDB	:	Protein Data Bank
PDT	:	Population Doubling Time
PGP	:	P Glycoprotein
PI	:	Propedium Iodide
PI3K	:	Phosphotidylionositol 3 Kinage
PR	:	Progesterone Receptor
QSAR	:	Quatitative Structure Activity Relationship
RMSD	:	Root Meansquare Deviation
RNA	:	Ribonucleic Acid
RPMI	:	RPMI (Rosewell Park Memorial Institute) Media 1640
SCID	:	Severe Comibined Immuno Deficiency
SFE	:	Sphere Formation Efficiency
SP	:	Side population
STR	:	Short Tandem Repeat
TDLU	:	Terminal duct-lobular unit
TEB	:	Terminal End Buds
TEL	:	Terminal End Buds
TEM	:	Transmission Electron Microscope
TGF-ß	:	Transforming Growth Factor-ß
TICs	:	Tumor Initiation Cells
TNBC	:	Triple Negative Breast Cancer
TNF	:	Tumor Necrosis Factor
TNM	:	Tumor Node Metastasis
TRAIL	:	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
UICC	:	Union of International Cancer Congress
VMD	:	Visual Molecular Dynamics

Chapter1

Introduction

Chapter 1: Introduction

Cancer is increasingly a global problem and breast cancer is not only the most common incident form of cancer in women worldwide, but is the first or second most common in all regions of the world, and responsible for 1.4 million new cases annually [1]. The incidence of breast cancer is increasing almost everywhere throughout the world, although the mortality rate from breast cancer is declining in many high income countries [2]. As per the ICMR-PBCR data, breast cancer is the commonest cancer among women in urban registries of Delhi, Mumbai, Ahmedabad, Kolkata, and Trivandrum where it constitutes >30% of all cancers in females (National Cancer Registry Programme, 2001). In the rural PBCR of Barshi, breast cancer is the second commonest cancer in women after cancer of the uterine cervix (National Cancer Registry Programme, 2001). The age standardized incidence rates (AARs) range from 6.2-39.5 per 100,000 Indian women. The AARs vary from region, ethnicity, religion, with the highest incidence reported at 48.3 per 100,000 women in the Parsi community of Mumbai (National Cancer Registry Programme, 2001).

Although the incidence of breast cancer has increased globally over the last several decades [3, 4]. The greatest increase has been reported in Asian countries. In Asia, breast cancer incidence peaks among women in their forties [5], whereas in the United States and Europe, it peaks among women in their sixties. In India premenopausal patients constitute about 50% of all patients [5]. Over 100,000 new breast cancer patients are estimated to be diagnosed annually in India [5, 6]. Breast cancer cases are expected to increase by 26% by 2020 and most of these will be seen in developing countries.

The incidence of this disease has been consistently increasing, and it is estimated to have risen by 50% between 1965 and 1985 [7]. The rise in incidence of 0.5-2% per annum has been seen across all regions of India and in all age groups but more so in the younger age groups (<45 years)[8]. A significant proportion of Indian breast cancer patients are younger than 35 years of age. This proportion varies between 11% at Tata Memorial Hospital, Mumbai [9] to 26% at SGPGIMS, Luck now [5]. Young age has been associated with larger tumour size, higher number of metastatic lymph nodes, poorer tumour grade, low rates of hormone receptor-

positive status, earlier and more frequent loco regional recurrences, and poorer overall survival [10, 11]. There is a significant difference in the survival rates in developed and developing countries mainly because of a lack of early detection programmes and inadequate resources for treatment. Coleman reported >80% survival from breast cancer in North America and Europe compared with 60% in middle-income countries and 40% in low-income countries [12].

Well characterized cell lines established directly from human tumors are powerful research resources for studying and comparing molecular and cellular processes, finding suitable molecular targets and development of new therapeutic strategies. Majority of breast cancer derived cell lines have been obtained from secondary tumours and pleural effusions of patients with advanced stage breast cancers [13-15]. Few breast cancer cell lines have been successfully established from primary tumours [16-18]. Recent studies and clinical investigations verify that the initially responsive tumors show relapse and resistance to one or multiple drugs used for the treatment. An emerging concept leading to chemoresistance is involvement of a less differentiated population in escaping the therapies. These cells called as cancer stem cells (CSCs) or tumor initiating cells or stem cell-like cells have certain properties similar to stem cells. The CSC theory asserts that many types of cancer are initiated from and maintained by a minor population of pluripotent cells having capability of continuous self-renewal and differentiation [19, 20]. This cell population undergoes unlimited proliferation and gives rise to differentiated cells, developing new tumors phenotypically recapitulating the original tumors [21]. In addition, recent studies indicate that CSCs may be responsible for tumor relapse and resistance to therapy [22, 23].

Evidence supporting the CSC model was initially obtained from acute myeloid leukemia (AML) [24, 25]. Subsequent studies support that solid tumors, including breast [26, 27], pancreatic [28, 29], brain [30, 31], colon [32-34], liver [35], head/neck [36], ovarian[37, 38], and melanoma [39, 40] are also driven and sustained by CSCs [25]. The first work in isolation and characterization of CSCs in solid tumors was conducted by Al-Hajj *et al.* Population of breast cancer cells expressing the surface marker, $CD44^{+}/CD24^{-/low}/Lin^{-}$, has capability to initiate tumors with the same heterogeneity as the primary tumor from 100 cells [27].

Similarly, enzymatic activity of aldehyde dehydrogenase 1 (ALDH) was also demonstrated to be a selective marker to enrich for breast cancer stem/progenitor cells [26]. These cell markers have been widely used to evaluate the ability of drugs to target cancer stem/progenitor cells [41-43].

Another technique that has been developed to isolate and characterize cancer stem/ progenitor cells is 'tumorosphere' culture [44-47]. This is based on the ability of stem/ progenitor cells to grow in serum-free, non-adherent suspension as spherical clusters, while differentiated cells fail to survive under the same condition [44, 45]. Cancer stem/progenitor cells are capable of yielding secondary spheres [44]. Decrease in tumorosphere formation in primary culture in the presence of drug treatment and in subsequent passages that are cultured in the absence of drugs indicate an inhibitory effect of the drug on self-renewal capacity of cancer stem/progenitor cells [41, 44].

Gaps in Existing Research and scope of the study:

In Indian women breast cancer appears to be different from western population as majority of breast cancer patients present with early onset, short disease-free interval and high mortality. There is high incidence of ER/PR negative breast cancers which are aggressive in nature and are not suitable for hormone therapy. So far, there is no breast cancer cell line available from Indian population in the international or national cell culture repository which limits the study of pathogenesis underlying breast cancer. Hence, there is a need to establish cell lines from primary breast tumors of Indian patients, which may be useful for understanding molecular mechanisms involved in breast tumourigenesis in Indian population. In view of the etiological and ethnic differences between the Indian and Western population these cells lines would be of considerable value to identify prognostic markers, drug targets and to establish prevention strategies.

The problems faced in management of tumors may be due to the fact that conventional treatments target the bulk of the tumor cells leaving behind the CSCs [48]. Anti-cancer therapies are effective at debulking the tumour mass but treatment effects are transient, with tumour relapse and metastatic disease often occurring as a result of the failure of targeting cancer stem cells [48]. For therapy

to be more effective, debulking of differentiated tumours must occur followed by targeting of the remaining surviving, often quiescent, tumour stem cells. Distinct expression levels and patterns of Cancer Stem Cell markers might be present in human breast carcinomas which might be responsible for their varied behaviour patterns and drug response. Also, the unique mechanisms or pathways associated specifically with the Cancer Stem Cells are not well understood. Studying cancer stem cell behaviour is important in understanding cancer pathogenesis. Since extensive self-renewal potential is the hallmark of stem cells, we undertook a detailed functional characterization of human BCSC mammospheres over long-term passages. Targeting Breast Cancer Stem Cells with novel drugs like Ellipticine and looking at its effect in combination with a well established anti-cancer drug like Paclitaxel on the proliferation and self renewal capacities of CSCs will give new insights into the possibility of a new combination chemotherapeutic regimen leading to improvements in the treatment of breast cancer.

Chapter 2

Aims and Objectives

Chapter 2: Aims and Objectives

1) Establishment and characterization of breast cancer cell lines from primary breast tumors in Indian women

2) Isolation and quantitation of cancer stem cells from breast cancer cell lines

3) To study the effect of anticancer drugs on breast cancer cell lines with high proportion of stem cells as compared to those with low proportion of stem cells

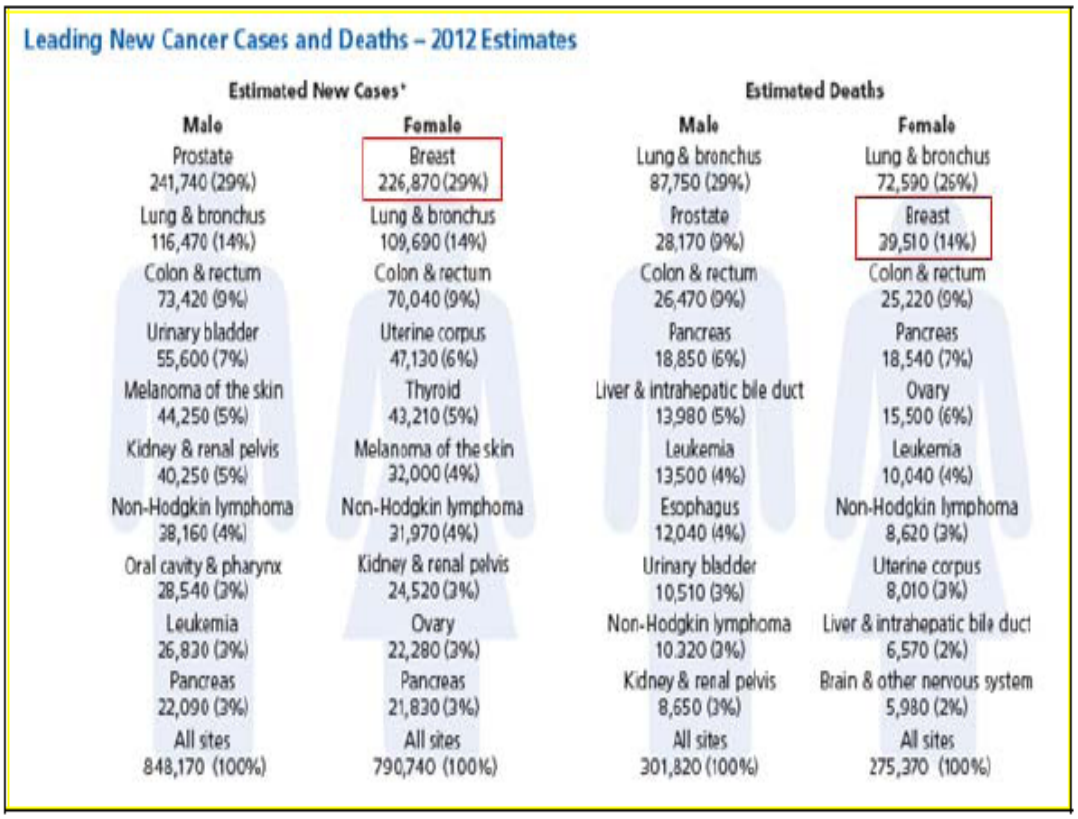
Chapter 3

Review of Literature

Chapter 3: Review of Literature

3.1. Global Scenario of breast cancer

The global burden of cancer continues to increase mostly because of the aging, factors like exposure to UV radiation, pollution and a rise in the adoption of cancer-causing behaviors viz., obesity, poor diet, smoking habits etc. Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among females [49].



(Adapted from: Cancer Facts & Figures 2011, Atlanta: American Cancer Society, 2011, copyright 2012 by American cancer society inc., surveillance research)

Figure 3.1. Statistical representation of cancer cases and deaths in the year 2012. Highlighted areas (Red bordered box) show number of breast cancer deaths and cases estimated in 2012.

Latest statistics estimated almost 230,480 new cases of the invasive breast cancer occurring among women during 2011 and about 2,140 new cases in men. For the

year 2012, almost 39,970 deaths due to breast cancer are expected along with 226,870 new cases (Figure 3.1) [50].

About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries. In general, incidence rates are high in Western and Northern Europe, Australia/New Zealand, and North America; intermediate in South America, the Caribbean, and Northern Africa; and low in sub-Saharan Africa and Asia (Figure 3.2).

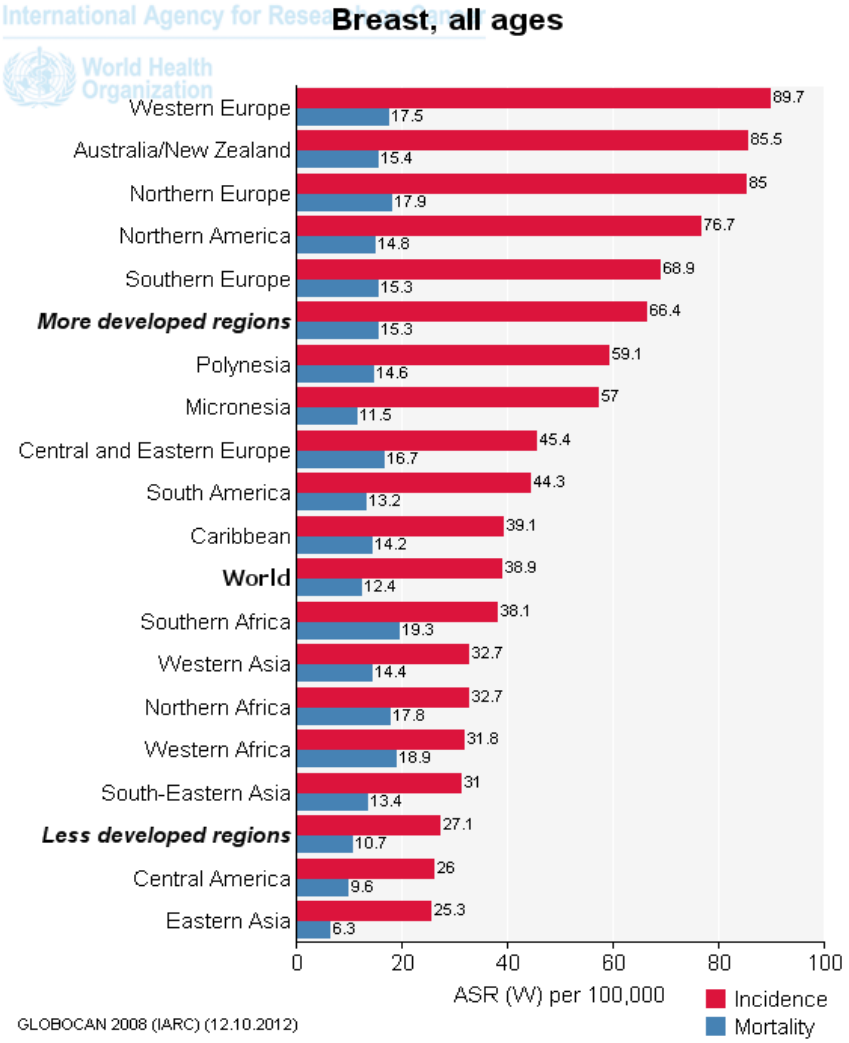


Figure 3.2 Worldwide Breast cancer Incidence and Mortality rates

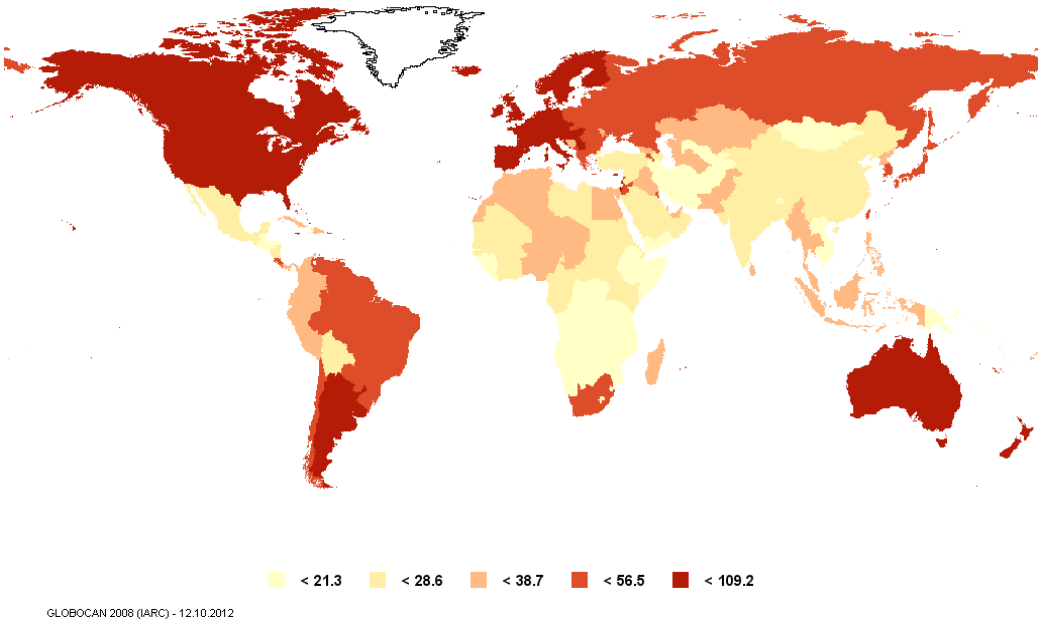


Figure 3.3. Estimated age-standardized incidence rate per 100,000 Breast, all ages

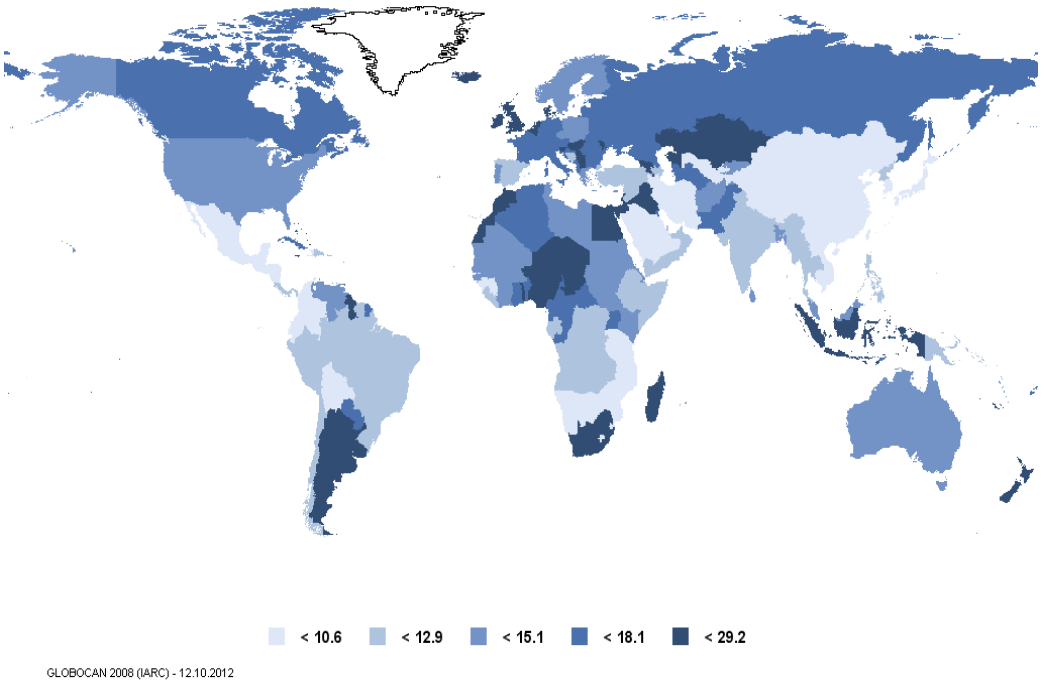


Figure 3.4 Estimated age-standardized mortality rate per 100,000 Breast, all ages

In contrast, breast cancer death rates have been decreasing in North America and several European countries over the past 25 years, largely as a result of early detection through mammography and improved treatment. In many African and Asian countries however, including Uganda, South Korea, and India, incidence and mortality rates have been rising (Figure 3.3 & 3.4) (Jemal, A et al. 2011).

3.2. Breast cancer scenario in India

Breast cancer is the second most common cancer among women in India, after cancer of the cervix uteri. In India, breast cancer contributes for 18.5% of the entire cancer burden. In the metropolitan cities, New Delhi and Mumbai, it is the most common cancer in women. In India, 75000 new cases of breast cancer occur annually [54].

Data from all urban and rural population-based cancer registries in India suggest a rising incidence of breast cancer in India. The age-adjusted rates reported from various urban registries range from 21.6 in Ahmadabad to 36.1 in Bangalore per 100,000, (Figure 3.5) which is about one-third the incidence reported from Western countries such as the United States (California SF: NH White, 109.6 per 100,000) and Uruguay (Montevideo, 114.9 per 100,000). The minimum age-adjusted rates reported from the rural population-based cancer registry in India are even lower (7.2 per 100,000) and somewhat similar to the incidence reported from other developing countries, such as The Gambia and Jiashan, China (NCRP INDIA 2006). The main factors responsible for increasing incidence could be India's urbanization and adoption of western lifestyle and food habits. Urban Indian women get married later, have fewer children, breastfeed them less, have a more western diet and higher alcohol intake leading to obesity which increases their lifetime exposure to estrogen and thereby their risk of developing the breast cancer. Although the risk of Indian women to develop breast cancer is significantly low than those in the western women but they are far more likely to die of it. Indian women have biologically aggressive breast cancer, as shown by lower incidence of estrogen receptor-positive, progesterone receptor-positive tumors and higher incidence of c-erbB2 [51].

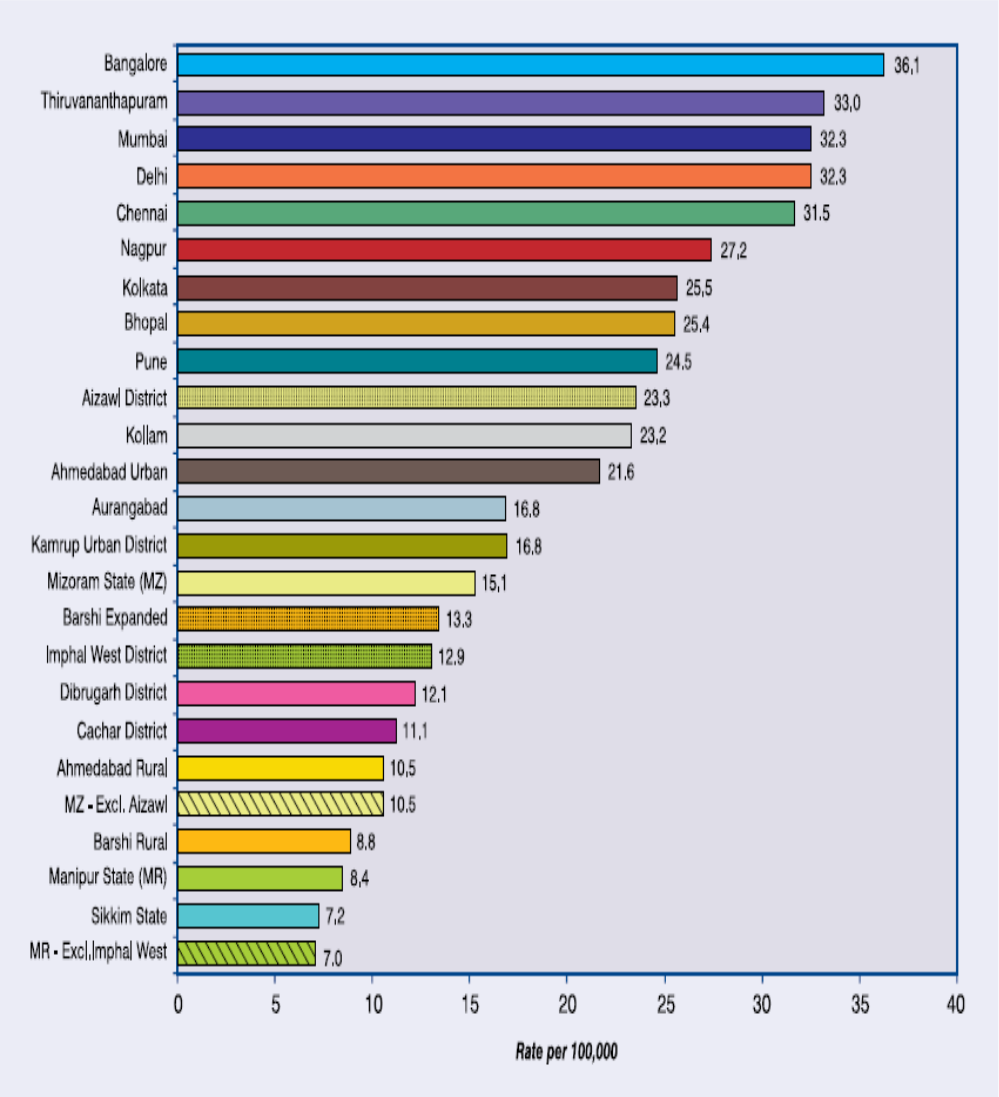


Figure 3.5 Comparison of Age Adjusted Breast cancer incidence rates (AARs) of all PBCRs

3.3. Normal anatomy of Breast

The breast or mammary gland is covered by skin and subcutaneous tissue [52] and rests on the pectoralis muscle, from which it is separated by a fascia. The morpho-functional unit of the organ is the single gland, a complex branching structure that is composed of two major parts: the terminal duct-lobular unit (TDLU) and the large duct system (Figure 3.6). The TDLU is formed by the lobule and terminal ductule and represents the secretory portion of the gland. Ducts lead to the nipple in the centre of a dark area of skin called the areola. Any type of malignant growth in TDLU or large duct in the breast tissue results in breast cancer.

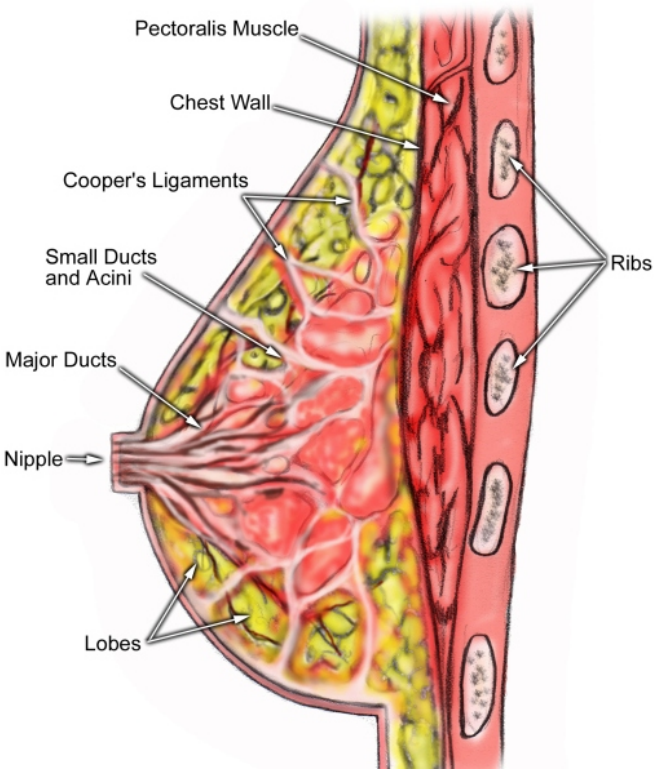


Figure 3.6 Anatomy of the human breast showing TDLU

3.3. Development of breast cancer

Numerous arguments support the hypothesis that tumors including breast carcinomas, arise from 1) stem cells, which possess many characteristics of cancer cells 2) accumulation of genetic/epigenetic changes and 3) from cells that are sustained during adult life. In human mammary gland, stem cells are thought to be located in the terminal ductal-lobular units (TDLU) or terminal end buds (TEL), the site from where most cancers originate [53-55]. A definitive mammary stem cell has not been identified and genes that determine its fate and factors that control its maintenance/proliferation remain yet unknown. In addition, based on their diverse phenotype, it's uncertain if breast carcinomas arise from a totipotent stem cell or from a 'transitory cell' with more restricted differentiation potential.

The natural history of breast cancer involves a sequential progression through defined clinical and pathologic stages starting with atypical hyperplasia to

carcinoma in situ and then invasive carcinomas that often culminate in metastatic disease (Figure 3.7) [56].

Tumor progression is driven by the sequential acquisition of various genetic and/or epigenetic changes in a single cell followed by clonal selection and expansion [57]. This mammary tumour progression model is strongly supported by human epidemiological studies as well as by studies in animal models of breast cancer. Ductal carcinoma in situ (DCIS) is believed to be the true precursor of invasive ductal carcinoma (IDC) based on its high rate of recurrence as an invasive tumor at its original site [58, 59].

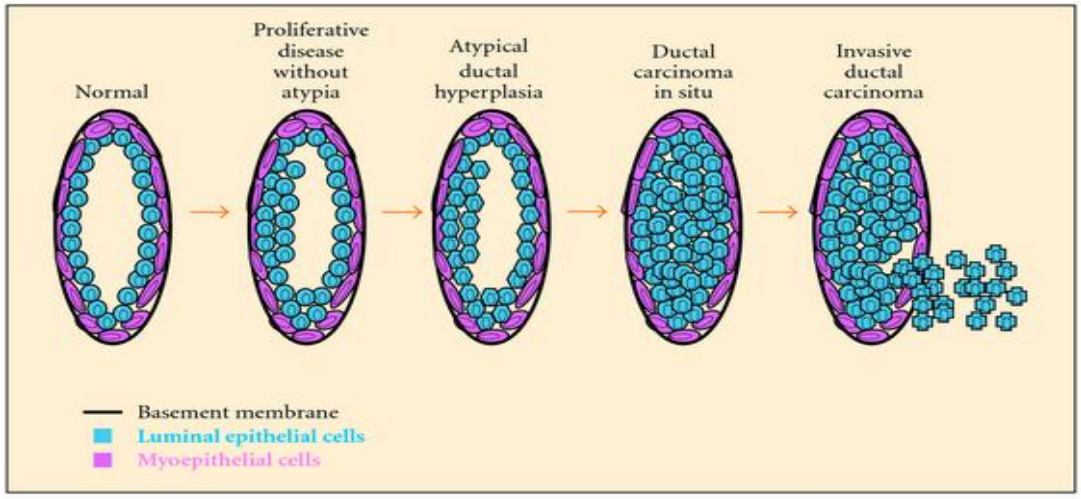


Figure 3.7 Progression of Breast Carcinoma

3.4. Morphological variants of breast cancer

Almost 95% of the breast cancers arise from epithelial cells and hence are termed carcinomas [60]. The two key determinations to make in the morphologic study of breast carcinoma are (i) whether the tumor is confined to the epithelial component of the organ (*in-situ*) or has invaded the stroma (invasive) and (ii) whether it is of ductal or lobular type.

***In-situ* Carcinoma**: The term in situ carcinoma is used to describe a proliferation of malignant epithelial cells that remain at their site of origin, confined by a basement membrane.

- **Ductal carcinoma *in-situ* (DCIS)** Tumor either arising from or involving a duct.
- **Lobular carcinoma in-situ (LCIS)** Tumor either rising from or involving a lobule.

Invasive breast cancer: Tumors included in this category are those in which stromal invasion is detectable, whether an *in-situ* component is identifiable or not and regardless of the relative proportion of the two components.

- **Invasive ductal carcinoma (IDC)** IDC accounts for the majority of invasive breast cancers. It begins in the cells forming the ducts of the breast. The gross appearance is usually typical with the irregular stellate outline.
- **Invasive lobular carcinoma (ILC)** It is characterized by the presence of small and relatively uniform tumor cells growing singly and in concentric (pagetoid) fashion around lobules involved by in situ lobular neoplasia.

Other types of breast cancer include inflammatory breast carcinoma, Paget's disease of the nipple, tubular, medullary, cribiform, metaplastic and apocrine carcinoma.

3.5. Clinical parameters of breast cancer

3.5.1. Staging of breast cancer

Staging refers to defining of cancerous growth according to extent of their disease, which is useful in determining the choice of treatment for individual patient, estimating their prognosis and comparing the results of different treatment programs. Staging is based on clinical as well as pathological findings. Currently, staging of cancer is determined by the parameters laid by American Joint Committee on Cancer (AJCC) 1977. The AJCC system is a clinical and pathologic staging system based on TNM nomenclature in which T refers to tumor, N refers to lymph node and M refers to metastases.

TNM

Classification

T= Primary Tumor

Tx - Primary tumor cannot be assessed

T0 - No evidence of primary tumor

Tis - Carcinoma in situ

Tis (DCIS) - Ductal carcinoma in situ

Tis (LCIS) - Lobular carcinoma in situ

T1 - ≤ 20 mm in greatest dimension

T2 - > 20 mm but ≤ 50 mm in greatest dimension

T3 - > 50 mm in greatest dimension

T4 - Tumor of any size with direct extension to the chest wall or skin

N= Regional Lymph Nodes

Nx - Regional lymph nodes cannot be assessed

N0 - No regional lymph node metastases

N1 - Metastases to movable ipsilateral axillary lymph node(s)

N2 - Metastases to ipsilateral axillary lymph node(s) fixed to one another or

other structure

N3 - Metastases to ipsilateral internal mammary lymph node(s)

M= Distant metastases

Mx - Presence of distant metastases cannot be assessed

M0 - No distant metastases

M1 - Distant metastases [including metastases to ipsilateral supraclavicular node(s)

Stage Grouping

Stage 0 - TisN0M0

Stage1 - T1N0M0

Stage IIA+ IIB - T0N1M0, T1N1M0, T2N0M0, T2N1M0, T3N0M0

Stage IIIA+ IIIB - T0N2M0, T1N2M0, T2N2M0, T3N1M0, T3N2M0+ T4 any N

M0, any T N3M0

Stage IV - any T any N M1

3.5.2. Grading of breast cancer

Histological grade is a widely accepted prognostic factor in invasive breast carcinoma. The Nottingham method is the most accepted method of determining the histological grade. The grade is obtained by adding up the scores for tubule formation, nuclear pleomorphism, and mitotic count resulting in a score of 3-9 points which is translated into the final grade by the following formula (Pathology), 3-5 points = Grade I; 6-7 points – Grade II; 8-9 points – Grade III.

3.6. Risk Factors for Breast Cancer

The most important risk factor for breast cancer is being female. Various other risk factors that affect the chance of getting Breast Cancer are detailed below.

3.6.1. Age

Age of the cancer patients is an important factor both for the occurrence and management of the disease.

Although the overall incidence of breast cancer in Indian population is low compared to Western population (ASR of 23.5 vs 90.7), the incidence of early

onset disease (<40 years) does not show significant variation (ASR range worldwide of 12-33) in different geographic regions [61] suggesting that in the Indian population a greater proportion of breast cancer is due to early onset disease compared to Western populations. According to the National Cancer Registry Project (NCRP) based on cancer registries at six hospitals, the average age of patients was found ranging between 44.2 years (Dibrugarh) to 49.6 years (Bangalore and Chennai) [62], whereas amongst US while females it is 61.0 years [63] showing that in Indian women, disease occurs a decade earlier than in Western populations.

3.6.2. Race

The incidence and mortality of breast cancer varies by race and ethnicity. In India, the incidence is more in urban than in rural women. The average incidence rate varies from 22-28 per 100,000 women per year in urban settings to 6 per 100,000 women per year in rural areas [62]. It is more prevalent in the higher socio-economic groups. Women of the Parsi community face a higher risk of breast cancer [64], than women of other religious communities. Ethnically isolated population of Northeast India is more prone to cancers compared to remaining part of the country. Certain pockets of North eastern region have highest breast cancer incidence rate like Aizwal district in Mizoram and Kamrup district in Assam [65].

3.6.3. Family history

Breast cancer risk is higher among women whose close blood relatives have this disease.[66-68]. History of cancers among family members helps in identifying high risk groups, who can be specifically counseled and subjected to careful follow ups with early diagnostic modalities and can even chose certain management approaches. It has been observed that familial breast cancer patients have an improved rate of survival, thereby indicating importance of noting familial association [7].

3.6.4. Reproductive factors

3.6.4.1.Age at menarche and menopause

Early age at menarche (less than 12 years of age versus more than 14 years of age) and delayed menopause (after age of 55 years) may slightly increase breast cancer risk, may be due to prolonged exposure of breast epitheliam to estrogen and progresteron.

3.6.4.2.Nulliparity and age at first birth

Nulliparity and older age at first birth contribute towards an increased risk of developing breast cancer [69, 70]. Nulliparous women had a 2.2-fold higher risk than multiparous women and late age at first pregnancy (30 years and above) showed excess risks of 5.4 compared with women having age at first pregnancy at 14 years or less [71] moreover, later age at first child birth emerged as stronger risk factor for contralateral breast cancer [72].

3.6.4.3.Breast feeding

Prolonged lactation has been demonstrated to be protective [73] for breast cancer development. There is a 4.3% decrease in the relative risk of breast cancer for every 12 months of breastfeeding, in addition to a decrease of 7.0% for each birth [74].

3.6.5. Lifestyle breast cancer risk factors

3.6.5.1.Alcohol and folate intake

A positive association between alcohol intake and the risk of developing breast cancer is observed in both pre and postmenopausal women with an overall risk of 1.6 [75]. For every 10g-increment (approx. 0.75-1 drink) increase in daily consumption of alcohol there is a 9% increase in risk [76]. Although the exact mechanisms has not been elucidated, it is found that alcohol can act indirectly through its first metabolite, acetaldehyde, a well-characterized carcinogen and mutagen, and/or can be a tumour promoter, leading to enhanced procarcinogen activation [77]. It has been demonstrated that increased folate intake may play a role in the prevention of breast cancer in women who consume alcohol [78].

3.6.5.2.Obesity and physical activity

Postmenopausal obesity increases the risk for developing breast cancer through higher levels of endogenous estrogen in obese women, as adipose tissue is an important source of estrogen and Progesterone [79]. Physical activity may reduce the risk by delaying the onset of menarche and modifying the bio available hormone levels [80].

3.6.6. Other breast cancer risk factors

3.6.6.1.Mammographic density

Breast density is an important predictor for breast cancer risk [81]. Two separate studies, Breast Cancer Detection Demonstration Project [82] and the Canadian National Breast Screening Study [83] have shown that women with more than 75% increased breast density on the mammography have an approximately five fold increase in the risk of developing breast carcinoma.

3.6.6.2.Ionizing radiation

Exposure of the mammary gland to high-dose ionizing radiation has been demonstrated to increase the risk of breast carcinoma [84, 85].

3.6.6.3. Steroid hormones

Breast cancer risk is enhanced by increasing the duration of exposure to endogenous ovarian hormones, so early menarche or late menopause increases the risk. Further, the risk of breast cancer is directly related to the age at which women bear first child. An early first, full-term pregnancy seems to have a protective effect. Women whose first pregnancy is delayed to their late 30s are at a higher risk than multiparous women. Unmarried women tend to be at a higher risk than married women. Further, nulliparity increases and high parity decreases the risk of breast cancer, at least after the age of 50. Exogenous hormonal factors such as estrogen replacement therapy and combined oral contraceptive use may cause a small increase in the risk for breast cancer [86, 87]. It has been hypothesized that progesterone may either decrease breast cancer risk, by mitigating the estrogen-induced proliferation of breast epithelial cells [88, 89], or increase risk because of the higher breast cell proliferation in the luteal phase [88] and increase the risk

associated with estrogen-plus progesterone hormone replacement therapy. Some studies have reported suggestive [90, 91] or statistically significant [92, 93] inverse, although not linear, associations between luteal progesterone levels and breast cancer risk.

3.6.6.4. Oral contraceptives

Oral contraceptives are associated with an increased risk of breast cancer. Grabrick *et al*., [94] observed a three fold increased risk of breast cancer among women who used oral contraceptives compared with those who never used oral contraceptives.

3.6.6.5. Benign breast diseases

Evidence from clinical follow-up studies has indicated that there is a relationship between the presence of histologically proven benign breast disease and breast cancer risk. In particular, proliferative lesions without atypia are associated with a 1.5- to 2-fold increase in risk, whereas atypical hyperplasia are associated with a fourfold to fivefold increase in breast cancer risk [95].

3.6.6.6. Genetic risk factors

3.6.6.6.1. p53 mutations in breast cancer

Wild type p53 is a tumor suppressor protein that plays a vital role in regulating genomic stability by controlling the cell cycle and inducing apoptosis when cell damage is beyond repair [96, 97]. Approximately 20% of all breast cancer cases have mutations in p53 [98]. An increased rate of p53 mutations affecting amino acids critical for DNA binding is reported in cancers arising in carriers of germ-line BRCA1 and BRCA2 mutations [99] Extensive research is being carried out to observe how various therapeutic approaches targeted to the p53 pathway impact on clinical outcome in breast cancer. There is evidence that specific mutations correlate with primary resistance to doxorubicin and that the presence of such mutations may be predictive of early relapse. In another study, cancers with p53 mutation were more likely to respond to paclitaxel. A number of innovative strategies are being studied to restore p53 function in tumours [57].

3.6.6.6.2. Defects in BRCA1

Defects in BRCA1 are a cause of genetic susceptibility to breast cancer. BRCA1 germline mutations are found in 40-50% of familial breast cancer cases. But somatic mutations of BRCA1 in sporadic breast carcinomas are rare [100, 101]. BRCA1 mutations are thought to be responsible for more than 80% of inherited breast-ovarian cancer cases. High grade ductal carcinomas are characterized by reduced protein expression of BRCA1 [100-102]. Complete loss of BRCA1 nuclear expression in breast cancer correlates with other poor prognostic markers like high histological grade, bcl-2-negativity and ER-negativity. BRCA1 expression may play an important role in the pathogenesis and prognosis of sporadic breast carcinoma. The reduced expression may be due to epigenetic or genetic alterations in promoter or due to other unknown mechanisms.

3.7. Establishment of in vitro cell cultures from breast carcinomas

Continuous cell lines derived from primary or metastatic cancers provide important experimental systems for studying the biology and genetic changes associated with tumour initiation and progression. Cell lines provide an unlimited, self-replicating source of cells that can be widely distributed to facilitate comparative studies. Since the establishment of the first human breast carcinoma cell line BT-20 in 1958 [103], many attempts have been made to establish permanent breast tumour cell lines. However, only limited success has been achieved in cultivating long-term epithelial cell cultures from human primary breast tumours [103-108]. Three distinct difficulties in establishing such cultures are 1. Rapid overgrowth of connective tissue, 2. Initial multiplication followed by death of tumour cells in primary cultures, and 3. failure of tumour cells to survive serial transfer [105, 107-110]. The difficulty in growing long term cultures arises because the traditional culturing methods using serum lead to overgrowth of fibroblasts which rapidly outnumber the epithelial elements. This can be minimized by the use of serum-free medium or by reduction of the concentration of serum to less than I% [111]. An additional problem is that the tumour frequently contains some normal breast epithelium which grows readily in tissue culture, and it is therefore difficult to determine whether the epithelium grown is malignant or benign [112]. To date the

majority of breast cancer derived cell lines have been obtained from secondary tumours and pleural effusions of patients with advanced stage breast cancers suggesting that cells from pleural effusions adapt better to their *in vitro* environment than cells from primary tumours [13-15, 113-118]. Few breast cancer cell lines have been successfully established from primary tumours [16-18, 119, 120]. Although about 100 human breast cancer lines have been described in literature, the number of breast tumour cell lines that have been adequately characterized and are widely used is only about 20 [121, 122]. Most in vitro studies using breast cancer cells are based on a few well characterized cell lines such as MCF7, T47D and MDA-MB-231 which have established in culture for over 20 years [123] (Table 3.1). The cultures established from secondary deposits (from which most of the available cultures were derived) are those collected late in the development of disease by which time there may have been a modification of the original tumour. In a study carried out by [123] McCallum and Lowther (1996) could establish 10 long term cell lines from 146 tumours with a success rate of 7%. Most successful cultures are those from grade III and ER and PR receptor negative tumors. Since loss of expression of estrogen receptor is associated with higher grade tumours that are more aggressive, probably cell lines may be established from such patients easily as compared to those of ER positive tumors. Most of the cell lines established from pleural effusions were negative for estrogen receptor. MCF-7 is the first among these shown to express estrogen receptor [114]. A relatively large panel of 21 paired tumour and normal breast cell lines was established from 189 patients with breast cancer using breast tumour specimens and their corresponding non-mour normal tissues [124]. These cell lines include a breast cancer cell line harbouring BRCA I germ-line mutation, HCC1937 [125].

Cell line	ER	PR	ERBB2/HER2	Source
184A1	-	NA	-	RM
BT20	-	-		PT
BT474	+	+	+	PT
BT483	+	+	-	PT
BT549	-	-	-	PT

CAL51	-	NA	-	PE
EFM19	+	+	-	PE
EFM192A	+	+	+	PE
HCC38	-	-	-	PT
HCC70	-	-	-	PT
HCC202	-	-	+	PT
HCC712	+	-	-	PT
HCC1007	+	-	+	PT
HCC1143	-	-	-	PT
HCC1187	-	-	-	PT
HCC1395	-	-	-	PT
HCC1419	-	-	+	PT
HCC1428	+	+	-	PE
HCC1500	+	+	-	PT
HCC1569	-	-	+	PT
HCC1599	-	-	-	PT
HCC1806	-	-	-	PT
HCC1937	-	-	-	PT
HCC1954	-	-	+	PT
HCC2157	-	-	-	PT
HCC2185	-	-	-	PE
HCC2218	-	-	+	PT
HCC2688	-	NA	-	PT
HCC3153	-	-	-	PT
HS578T	-	-	-	PT
hTERT-HME1	-	NA	-	RM
MCF7	+	+	-	PE
MCF10A	-	-	-	RM
MDA134	+	-	-	PE
MDA157	-	-	-	PE

MDA175	+	-	-	PE
MDA231	-	-	-	PE
MDA361	+	+	+	BR
MDA436	-	-	-	PE
MDA453	-	-	+	PE
MDA468	-	-	-	PE
SKBR3	-	-	+	PE
SUM44	+	+	+	PE
SUM52	+	-	+	PE
SUM102	-	-	-	PE
SUM149	-	-	-	PE
SUM159	-	-	-	PT
SUM190	-	-	+	PT
T47D	+	+	-	PE
UACC812	+	-	+	PT
UACC893	-	-	+	PT
ZR75-1	+	-	-	AF
ZR75-30	+	-	+	AF

Table 3.1 Clinicopathological features of some well characterized breast cancer cell lines.

3.8. Breast cancer cell lines as models

3.8.1. Cell lines in xenograft studies

Whilst xenograft models provide a whole organism environment for tumour growth, these too have limitations. Experiments are usually performed in immunocompromised mice, which can impact on tumour formation and progression. The site of implantation is an important consideration, with injections into the mammary fat pad considered more physiologically relevant than subcutaneous injections even though the mouse and human mammary glands have quite different structures. Another confounding variable is the distinct difference between the stroma of human and mouse mammary tissue, which casts doubt on

the relevance of xenograft models [52]. As discussed above, the stroma is now recognised to influence breast tumour cells. The differring biology of mouse and human stroma together with reports of spontaneous transformation of mouse stroma by human breast tumour xenografts, resulting in hybrid mouse–human nuclei within the xenograft [53], raise further concerns. Several groups have tried to overcome this by co-injecting human fibroblasts with cancer cell lines [54, 55], but this does not allow for co-evolution of tumour and stroma that would happen during cancer development. Of the cell lines commonly incorporated into xenograft models, ER-positive luminal A cell lines such as MCF-7 and T47D will only form tumours in the presence of oestrogen and, unsurprisingly, growth can be inhibited by anti-oestrogen therapy. Cell lines representing other subtypes (for example, BT474, MDA-MB-468 and MDA-MB- 231) have also been shown to be tumourigenic; however, cells representing the HER2 subtype, including SKBR3 and MDA-MB-453 cells, have poor tumourigenic potential. An unexpected finding with xenograft models is the limited ability of tumours to invade and metastasise, particularly given the often metastatic origin of cell lines (reviewed in [14]). If metastasis occurs it is usually to the lung, which is not the most common metastatic site in human breast cancer – thus breast cancer metastasis is often studied through intravenous injection, enabling colonisation of specific organs; for example, intracarotid artery injection for study of brain metastasis or left ventricle injection for metastasis to bone. Cell lines such as MDA-MB-231 that are regarded as invasive *in vitro* remain relatively poorly metastatic *in vivo*, although when introduced directly into the circulation the cell line has proved useful in models of experimental metastasis. Using the human breast cancer cell line SUM1315 derived from a clinical sample of a metastatic node, Kupperwasser and colleagues introduced this as an orthotopic model into immunodeficient (NOD/SCID) mice bearing grafts of human bone, and showed the cells preferentially and spontaneously metastasised to the human bone graft rather than mouse skeleton [57].

3.8.2. Cell lines as hormone-unresponsive models

The majority of breast cancer cell lines are ER- negative. Consequently they are considered hormone unresponsive both in vitro and in vivo. Most of the ER-

negative xenografts produce poorly differentiated adenocarcinomas, relative to the ER-positive xenografts. Several are highly locally invasive. Clarke et al., recently generated an ascites variant of the MDA-MB-435 cell line [120]. These cells, designated MDA-435/LCC6, appear to have arisen from a locally invasive mammary fat pad tumor that invaded into the peritoneal cavity. The ascites can be maintained either in vivo, or as a monolayer culture in vitro, and have a highly reproducible duration of survival from cell inoculation to morbidity/death. These cells provide a novel model in which to study the pathogenesis of malignant ascites and the effect of in vitro [120] or in vivo gene transfer by retro viral vectors [121]. The MDA-435/LCC6 ascites variants grow equally well in nu/nu mice and rnu/rnu rats [122].

3.8.3. Breast cancer cell lines as drug resistant models

Probably the most widely used breast cancer model of drug resistance is the $MCF7^{ADR}$ cell line. These cells were stepwise selected against doxorubicin in vitro, and overexpress the gp170 product (P-glycoprotein, PGP) of the human MDR1 gene. $MCF7^{ADR}$ cells also have become ER-negative and resistant to antiestrogens [123]. Despite its frequent use to screen gp170-reversing agents, this cell line expresses several other potential multiple drug resistance mechanisms. $MCF7^{ADR}$, but not MDR1-transduced MCF7 cells (CL 10.3), are cross resistant to Tumor Necrosis Factor [124], an observation that indicates the presence of ADR resistance mechanisms in addition to gp170. While this would include changes in manganous superoxide dismutase expression/activity [124], the activities of glutathione transferase and topoisomerase II respectively also are altered in $MCF7^{ADR\ cells}$

3.8.4. Breast cancer cell lines as models of mammary cancer stem cells

Stem cells are characterised by their ability to yield new tumours when xenografted into immunodeficient mice. Breast CSCs are identified by one or more of the following features: their ability to form tumours *in vivo*; mammosphere formation *in vitro*; expression of aldehyde dehydrogenase; or through expression of cell surface biomarkers, usually the CD44+/CD24–/low phenotype [30]. There are clearly many advantages to working with CSCs derived from cell lines as they

may be good models to further understand stem cell biology and develop CSC-specific therapeutic targets. Two major obstacles need to be overcome, however, before these can be developed for routine use: CSCs are very much in the minority within a given tumour population, and CSCs have extremely slow population-doubling times. Improved enrichment methods are required to provide sufficient numbers of CSCs to conduct these types of studies, and their slow proliferation rates are challenging when it comes to experimentally testing potential new therapeutics.

3.9. Breast cancer treatment, resistance and tumor recurrence

There are several chemotherapy drugs that are commonly used (Table 3.2), with multiple combinations, even though basic action of most of the drugs is on interfering the cell division. Many of these drugs are derived from plants and execute their therapeutic and/or chemoprotective functions through multiple pathways (Figure 3.8).

Chemotherapeutic Drug	**Brand name**
Paclitaxel	Abraxane
Doxorubicin	Adriamycin, Doxil
Carboplatin	Paraplatin
Cyclophosphamide	Cytoxan
Daunorubicin	Cerubidine, DaunoXome
Epirubicin	Ellence
Fluorouracil	Adrucil
Gemcitabine	Gemzar
Eribulin	Halaven
Ixabepilone	Ixempra
Methotrexate	Amethopterin, Mexate,
Mutamycin	Mitomycin
Mitoxantrone	Novantrone

Vinorelbine	Navelbine
Docetaxel	Taxotere
Thiotepa	Thioplex
Vincristine	Oncovin, Vincasar PES, Vincrex
Xeloda	Capecitabine

Table 3.2 Chemotherapy medications for breast cancer

Some drugs acts by disrupting microtubule formation (Taxane derivatives ex: Paclitaxel) [126], by acting as antimetabolites (Flurouracil) [127][128] and DNA altering agents which act by intercalating DNA (Doxirubicine, antracyclines etc) [129-131]. In addition, hormonal therapies that blocks Estrogen receptors (tamoxifen and fulvestrant) and inhibit estrogen formation itself (anastrozole) are also being used in the breast cancer treatment. But for reasons that are still unclear, patients often develop resistance to these therapies, especially when their tumors migrate—metastasize—to other organs.

Standard chemotherapy regimens:

- AT: Adriamycin and Taxotere
- AC ± T: Adriamycin and Cytoxan, with or without Taxol or Taxotere
- CMF: Cytoxan, methotrexate, and fluorouracil
- CEF: Cytoxan, Ellence, and fluorouracil
- CAF: Cytoxan, Adriamycin, and fluorouracil
- TAC: Taxotere, Adriamycin, and Cytoxan
- GET: Gemzar, Ellence, and Taxol

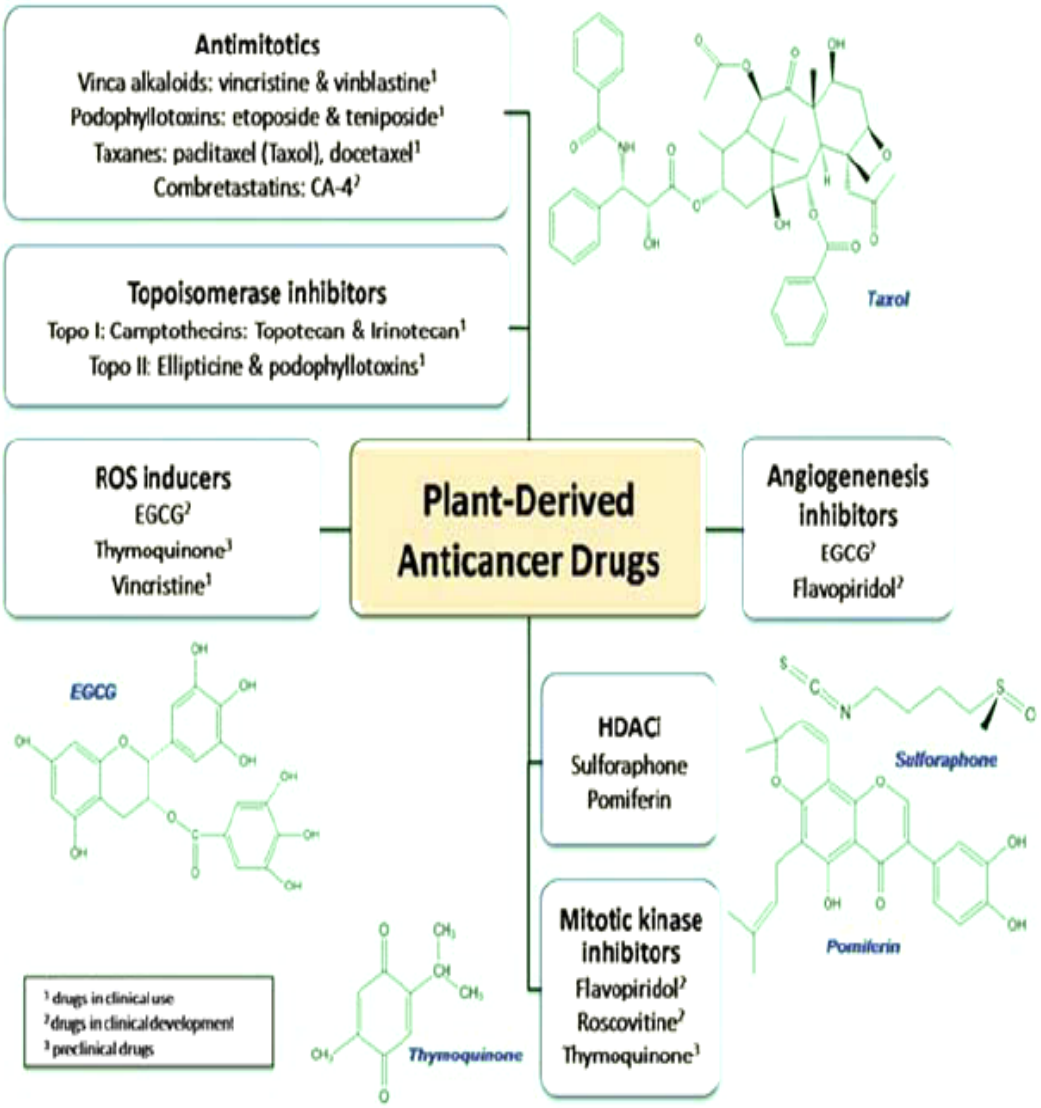


Figure 3.8. Selected groups of plant-based anticancer drugs. Some drugs may execute their therapeutic and/or chemoprotective functions through multiple pathways.

3.10. Cancer stem cells (CSCs)

The CSC theory asserts that many types of cancer are initiated from and maintained by a minor population of tumorigenic cells that are capable of continuous self-renewal and differentiation [19, 20] (Figure 3.9). This cell population undergoes unlimited proliferation and gives rise to differentiated cells, developing new tumors phenotypically recapitulating the original tumors [21]. In addition, recent studies indicate that CSCs may be responsible for tumor relapse and resistance to therapy [22, 23].

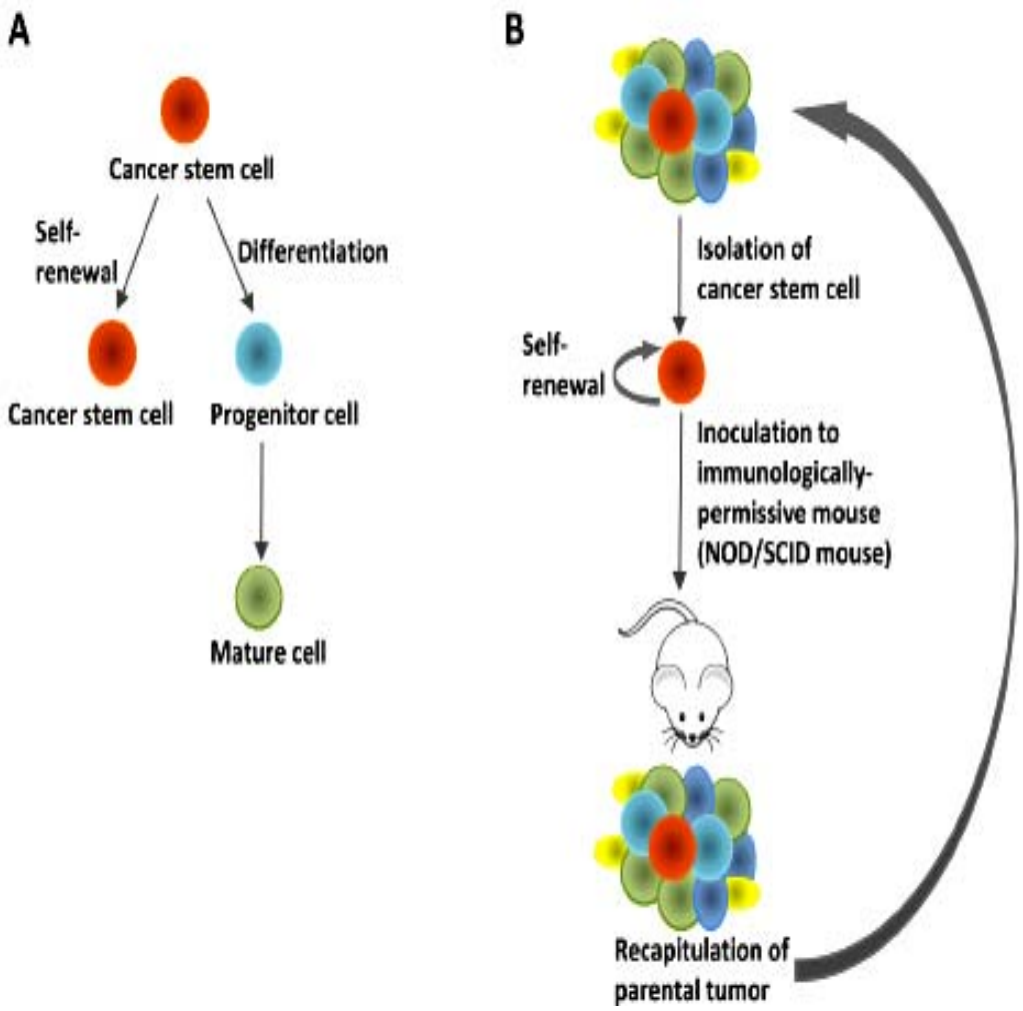


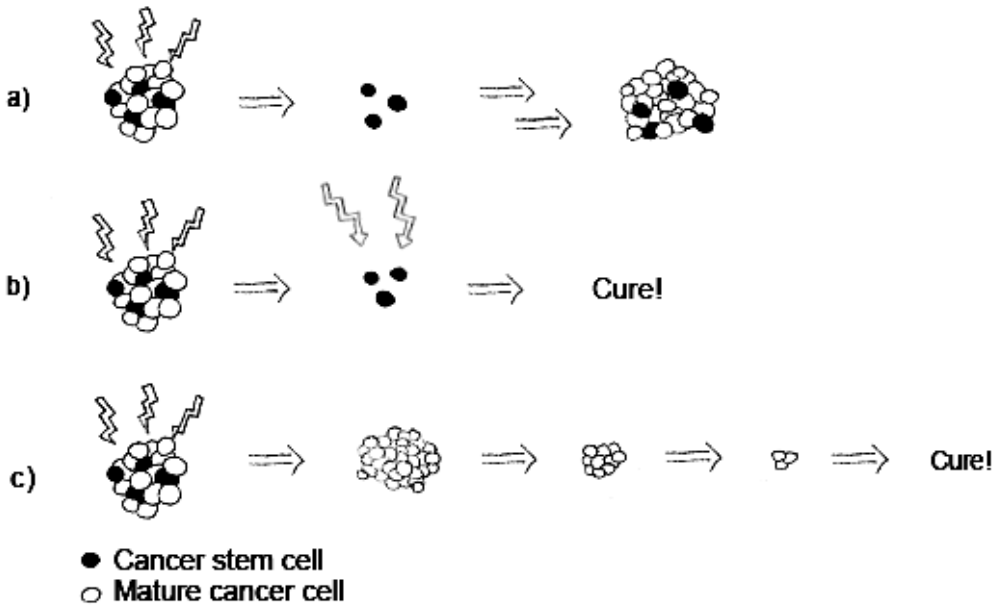
Figure 3.9 Cancer stem cell theory. (A) Cancer stem cells are capable of self-renewal and differentiation. (B) Isolated cancer stem cells are able to phenotypically recapitulate the parental tumor along serial passaging through multiple recipient mice.

Evidence supporting the CSC model was initially obtained from acute myeloid leukemia (AML) [24, 25]. Subsequent studies support that solid tumors, including breast [26, 27], pancreatic [28, 29], brain [30, 31], colon [32-34], liver [35], head/neck [36], ovarian [37, 38], and melanoma [39, 40] are also driven and sustained by CSCs [25]. The first work in isolation and characterization of CSCs in solid tumors was conducted by Al-Hajj *et al.* A breast cancer cell population expressing the surface marker, $CD44^{+}/CD24^{-/low}/Lin-$, was able to initiate tumors with the same heterogeneity as the primary tumor from 100 cells [27].

Similarly, enzymatic activity of aldehyde dehydrogenase 1 (ALDH) was also demonstrated to be a selective marker to enrich for breast cancer stem/progenitor cells [26]. These two phenotypes, ALDH-positive and CD44+CD24−/low/Lin−, were identified as possessing a small overlap that has the highest tumorigenic capacity, generating tumors from as few as 20 cells [26]. Recently, the CD44+CD24+ESA+ and CD133+ subpopulations were found to harbor putative pancreatic CSCs [28, 29], and an overlap was suggested to exist between these two populations [29]. These cell markers have been widely used to evaluate the ability of drugs to target cancer stem/progenitor cells [41-43].

3.10.1. Involvement of cancer stem cells (CSCs)

An emerging concept leading to chemoresistance is involvement of a less differentiated population in escaping the therapies. These cells called cancer stem cells or tumor initiating cells or stem cell-like cells have certain properties related to stem cells. One of the properties of these cells is “quiescence” or slow proliferation that confers resistance to chemotherapeutics [132] (Figure. 3.10).



Adapted from Dalarba et al., 2007.

Figure 3.10 Chemoresistance of Cancer stem cells. (a) Treatment not targeting CSCs. In time tumorigenesis may continue leading to relapse. (b) Traditional treatment accompanied by CSC- specific treatment may lead to cure. When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells (data not shown). Thus, we hypothesize that lack of mammosphere formation could not be attributed to the absence of live cells beyond the sixth/seventh passage, but due to differentiation of cancer stem cells. (c) CSC-specific treatment with initially little effect on tumor size may gradually lead to shrinkage of the tumor as the mature cancer cells are not able to sustain tumor growth.

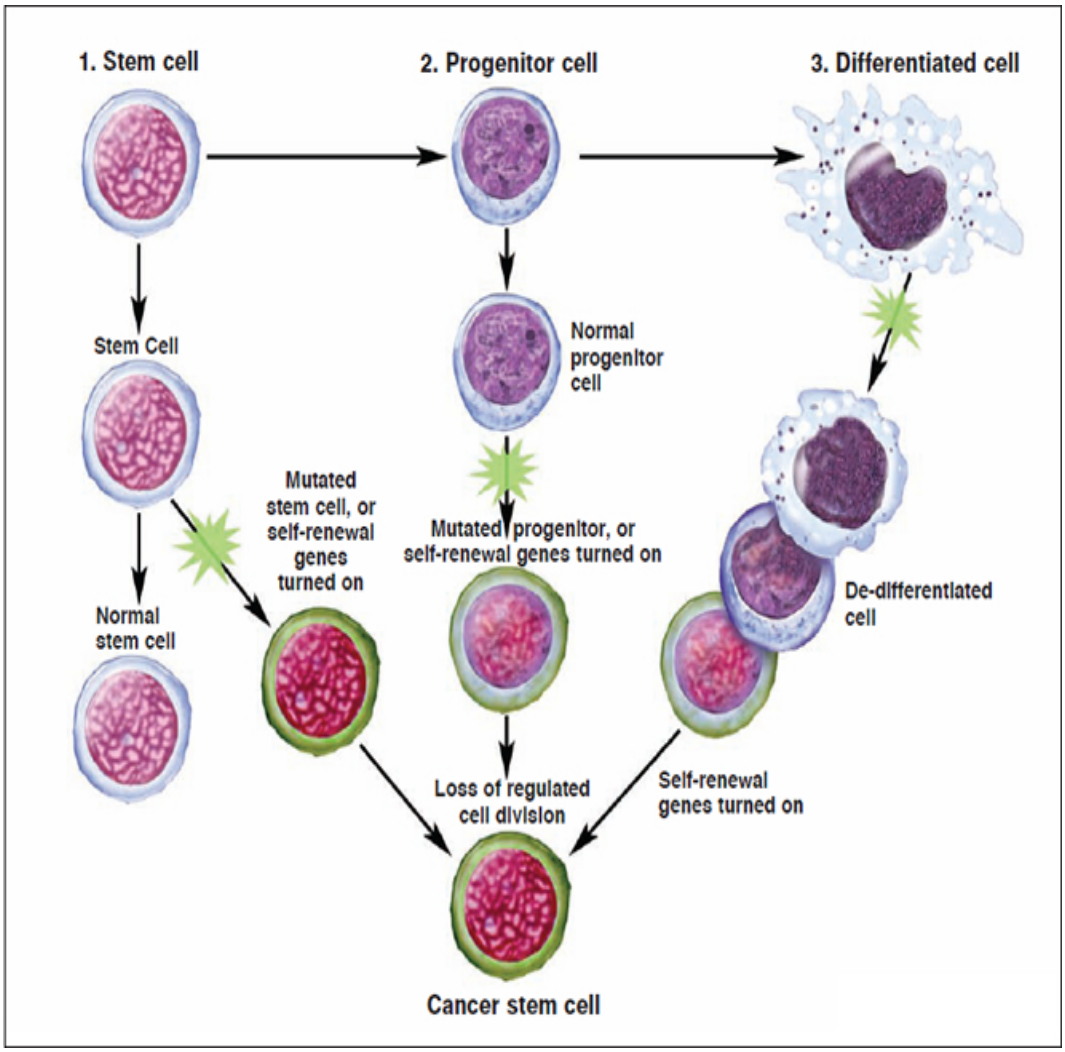
3.10.2. Origin of cancer stem cells

Given the similarities between tumor-initiating cells and stem cells, researchers have sought to determine whether CSCs arise from stem cells, progenitor cells, or differentiated cells present in adult tissue. Of course, different malignancies may present different answers to this question. The issue is currently under debate and several theories about the cellular precursors of cancer cells have been proposed. (Figure 3.11).

Hypothesis #1: Cancer cells arise from stem cells.

Stem cells are distinguished from other cells by two characteristics: (1) they can divide to produce copies of themselves, or 'self-renew', under appropriate conditions and they are 'pluripotent', or [46] able to differentiate into most, if not all, mature cell types. If CSCs arise from normal stem cells present in the adult tissue, de-differentiation would not be necessary for tumor formation. In this scenario, cancer cells could simply utilize the existing stem-cell regulatory pathways to promote their self-renewal. The ability to self-renew gives stem cells long lifespans relative to those of mature, differentiated cells [133]. It has therefore been hypothesized that the limited lifespan of a mature cell makes it less likely to live long enough to undergo the multiple mutations necessary for tumor formation and metastasis.

Several characteristics of the leukemia-initiating cells support the stem-cell origin hypothesis. Recently, the CSCs associated with AML have been shown to comprise distinct, hierarchically-arranged classes (similar to those observed with **hematopoietic stem cells**) that dictate distinct fates [134]. To investigate whether these CSCs derive from hematopoietic stem cells, researchers have used a technique known as serial dilution to determine the CSCs' ability to self-renew. Serial dilution involves transplanting cells (usually hematopoietic stem cells, but in this case, CSCs) into a mouse during a bone-marrow transplant. Prior to the transplant, this "primary recipient" mouse's natural supply of hematopoietic stem cells is ablated. If the transplant is successful and if the cells undergo substantial self-renewal, the primary recipient can then become a successful donor for a subsequent, or serial, transplant. Following cell division within primary recipients, a subset of the AML-associated CSCs divided only rarely and underwent self-renewal instead of committing to a lineage. This heterogeneity in self-renewal potential supports the hypothesis that these CSCs derive from normal hematopoietic stem cells [134]. It should be noted, however, that the leukemia-inducing cells are the longest-studied of the known CSCs; the identification and characterization of other CSCs will allow researchers to understand more about the origin of these unique cells.



Adapted from NIH, 2010

Figure 3.11 Rise of cancer stem cells. The molecular pathways that maintain "stem-ness" in stem cells are also active in numerous cancers. This similarity has led scientists to propose that cancers may arise when some event produces a mutation in a stem cell, robbing it of the ability to regulate cell division. This figure illustrates 3 hypotheses of how a cancer stem cell may arise: (1) A stem cell undergoes a mutation, [46] A progenitor cell undergoes two or more mutations, or (3) A fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all 3 scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division.

Hypothesis #2: Cancer cells arise from progenitor cells.

The differentiation pathway from a stem cell to a differentiated cell usually involves one or more intermediate cell types. These intermediate cells, which are more abundant in adult tissue than are stem cells, are called progenitor or precursor cells. They are partly differentiated cells present in foetal and adult tissues that usually divide to produce mature cells. However, they retain a partial capacity for self-renewal. This property, when considered with their abundance relative to stem cells in adult tissue, has led some researchers to postulate that progenitor cells could be a source of CSCs [135, 136].

Hypothesis #3: Cancer cells arise from differentiated cells.

Some researchers have suggested that cancer cells could arise from mature, differentiated cells that somehow de-differentiate to become more stem cell-like. In this scenario, the requisite oncogenic (cancer causing) genetic mutations would need to drive the de-differentiation process as well as the subsequent self-renewal of the proliferating cells. This model leaves open the possibility that a relatively large population of cells in the tissue could have tumorigenic potential; a small subset of these would actually initiate the tumor. Specific mechanisms to select, which cells would de-differentiate have not been proposed. However, if a tissue contains a sufficient population of differentiated cells, the laws of probability indicate that a small portion of them could, in principle, undergo the sequence of events necessary for de-differentiation. Moreover, this sequence may contain surprisingly few steps; researchers have recently demonstrated that human adult somatic cells can be genetically "re-programmed" into pluripotent human stem cells [137, 138].

3.10.3. Self-renewal pathways of cancer stem cells

Understanding the mechanisms that underlie the self-renewal behavior of CSCs [20, 139] is important for discovery and development of anti-cancer drugs targeting CSCs. Several pathways have been identified to play pivotal roles in CSC self-renewal [140-142].

1. Wnt/β-catenin pathway

Wnt/β-catenin pathway was demonstrated to modulate cell proliferation, migration, apoptosis, differentiation, and stem cell self-renewal [143-146]. It has been shown that Wnt/β- catenin signaling is implicated in the maintenance of CSCs of leukemia [147-149], melanoma [150], breast [151, 152], colon [153], liver [154], lung [155] cancers. Over-expression of β-catenin in stem cell survival pathway was shown to mediate the resistance of mouse mammary stem/progenitor cells to radiation [152]. Elimination of β-catenin abrogated the chemo-resistant cell population endowed with progenitor-like features [154]. The link between Wnt/β-catenin and PI3K/Akt pathway has been established by several studies. Korkaya *et al.* demonstrated that PI3K/Akt pathway is important in regulating the mammary stem/progenitor cells by promoting β-catenin downstream events through phosphorylation of Ser9 on GSK3β [19] [156-158].

2. Hedgehog pathway

Hedgehog pathway plays a crucial role in regulating self-renewal of normal and malignant human mammary stem cells [45, 140, 159, 160]. When secreted hedgehog ligands bind to Ptch, Smo is released, triggering dissociation of transcription factors, Gli1, Gli2, and Gli3 from Fused and suppressor of Fused (SuFu), leading to transcription of an array of genes, such as cyclin D, cyclin E, Myc, and elements of EGF pathway [159, 161-163]. It was suggested that over-expression of sonic hedgehog is activated by NF-κB in pancreatic cancer thereby enhancing cell proliferation [164].

3. Notch pathway

Notch signaling is known to control cell proliferation and apoptosis to modulate the development of many organs [165]. Notch pathway is believed to be dysregulated in CSCs, ultimately leading to uncontrolled CSC self-renewal [165], of malignant breast cancer CSCs [141, 166]. Specifically, Notch-1 is necessary for expression of several NF-κB subunits [167, 168] and stimulates NF-κB promoter activity [167]. Notch proteins are expressed as transmembrane receptors in a

variety of stem/progenitor cells [169]. Binding of surface-bound ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4) triggers serial cleavage events at the Notch proteins by ADAM protease family and γ-secretase [169-171]. Subsequently, the intracellular domain of Notch is released and translocates into the nucleus, where it acts as a transcription co-activator of recombination signal sequence-binding protein Jκ (RBP-J) to activate downstream target genes, e.g., c-Myc, cyclin D1, p21, NF-κB [171-177].

3.10. Targeting self-renewal pathways of cancer stem cells by natural dietary compounds

Since CSCs are more resistant to conventional therapies in comparison with differentiated cells constituting the tumor bulk, combination of drugs that are directed against CSCs and conventional chemotherapy would have the potential to overcome tumor resistance, reduce relapse [20], CSCs can be targeted by several strategies including sensitizing them to chemotherapeutic agents, induction of differentiation, and inhibition of self-renewal signaling [21, 178]. Number of studies have found that some dietary compounds can directly or indirectly affect CSC self-renewal pathways [178] (Table 3.3).

Natural dietary Compound	Food Origin	Cancer Stem Cell	Elements of self-renewal pathways
Curcumin	Turmeric	Pancreatic cancer stem cells, breast cancer stem cells	β-catenin, TCF-4, Frizzled-1; Notch-1
Sulforaphane	Cruciferous vegetables		B-catenin. GSK3β(?), Wnt9a
Soy isoflavone (especially genistein)	Soy		GSK3β, β-catenin, E-cadherin, Wnt-5a, Sfrp-2; Notch-2

Epigallocatechin-3-gallate	Green tea		HBP 1, β-catenin, GSK3β(?)
Resveratrol	Grapes, berries, plums and peanuts		β-catenin, GSK3β; Notch-1
Lycopene	Tomatoes, watermelon, papaya, pink grapefruit		β-catenin, GSK3β(?)
Piperine	Black and long pepper	Breast cancer stem cells	Wnt/ β-catenin
Vitamin D3			TCF-4, E-cadherin

Table 3.3 Natural dietary compounds that selectively regulate cancer stem cell self-renewal and inhibit cancer stem cells.

1. Curcumin

Curcumin, a polyphenol present in turmeric [179], possesses anti-inflammatory and anti-oxidant activities [179, 180], and has been studied as a chemoprevention agent in several cancer models [181, 182]. Curcumin induced caspase-3- mediated cleavage of β-catenin, leading to inactivation of Wnt/β-catenin signaling [183], and decreased β-catenin/TCF transcription activity in all tested cancer cell lines [179]. In addition, curcumin down-regulated Notch-1 mRNA level in pancreatic cancer cells, indicating a transcriptional inactivation of Notch-1 [184]. Curcumin-induced inactivation of NF-κB DNA-binding activity was potentially mediated by Notch-1 signaling pathway [184]. Also, Kakarala *et al.,* demonstrated that curcumin was able to target breast stem/ progenitor cells [185].

2. Sulforaphane

Sulforaphane, which is converted from a major glucosinolate in broccoli/broccoli sprouts [186], could abrogate the resistance of pancreatic TICs to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) by interfering with TRAIL-activated NF-κB signaling [52]. The down-regulation of NF-κB function by sulforaphane treatment has been reported in prostate and colon cancer cells as well [187-189]. Several studies have reported the activity of sulforaphane to down-regulate β-catenin and Akt pathway in ovarian, prostate, and colorectal cancers [190-192]. Wicha *et. al.*, have shown that sulforaphane is effective in targeting breast cancer stem/ progenitor cells *in vitro* and *in vivo* [41]; and down regulates Wnt/β-catenin self-renewal pathway in breast cancer cells.

3. Soy isoflavone

Increased plasma concentration of genistein (one of the most active soy isoflavones) due to soy food intake was associated with reduced risk of breast cancer in recent studies [193, 194]. Soy isoflavones, especially genistein, exhibit potent anti-proliferative effect on various cancers [195]. Soy isoflavones were found to inhibit the phosphorylation of Akt and FOXO3a, enhance the expression of GSK3β, leading to increased phosphorylation of β-catenin in prostate cancer cells [196, 197].

4. Epigallocatechin-3-gallate (EGCG)

Epigallocatechin-3-gallate (EGCG), a catechin from green tea, is a potent chemoprevention agent [198]. EGCG has been shown to inhibit NF- κB activity, MAPK pathway, activator protein-1 (AP-1) activity, and EGFR-mediated downstream signaling pathways, etc. [199]. EGCG was demonstrated to block Wnt signaling [178, 200] and suppress Akt activation in both colon cancer cell lines and *in vivo* mouse models [199, 201-203].

5. Resveratrol

Resveratrol, a polyphenol derived from a wide variety of plants such as grapes, berries, plums, and peanuts [204], has been shown to possess inhibitory effect on the proliferation of various human cancer cells and on the carcinogenesis in animal

models [205]. Resveratrol has been shown to significantly decrease the nuclear localization of β-catenin [206] and inhibite the PI3K/Akt pathway, thereby activating GSK3β in colon cancer and acute lymphoblastic leukemia cells [207].

6. Lycopene

Lycopene, one of the most extensively studied carotenoids in tomatoes has been shown to induce apoptosis and inhibit cell cycle progression in various cancer cells [208-214]. In colon cancer cells, lycopene suppressed Akt activation and non-phosphorylated β-catenin protein level, and augmented the phosphorylated form of β-catenin, which were associated with reduced protein expression of cyclin D1 [215].

7. Piperine

Piperine, a dietary polyphenol isolated from black and long peppers, has been reported to reduce cancer incidence in chemical rodent models of lung cancer [185, 216-219]. Although the chemoprevention effect of piperine in breast cancer as a single agent has not been explored, Kakarala *et al.* demonstrated that piperine was able to target breast CSCs and inhibit Wnt/β-catenin signaling pathway [185].

8. Vitamin D3

Vitamin D3 has been shown to reduce the incidence of human breast, prostate, and colon cancers [220-222] and induce apoptosis and cell cycle arrest of various cancer cells [223]. In 2001, Palmer *et al.* demonstrated that vitamin D3 promoted the differentiation of colon carcinoma cells by the induction of E-cadherin expression and the inhibition of β-catenin signaling [224].

3.11. Techniques for the characterization of cancer stem cells

3.11.1. Side population technique

The SP technique is based on the abilities of stem cells to exclude vital dyes Hoechst 33342 or Rhodamin 123 [225-228]. Normal and cancer stem cells express transmembrane transporters, such as the ATP-binding cassette protein, ABC transporter ABCG2/BCRP1 (breast cancer resistance protein 1). The SP technique for CSCs has also been successfully used in several cell lines and tissues [229,

230] [225-228, 231]. However, functional studies using Hoechst staining are limited by the toxicity of this agent. Furthermore, evidence from mouse models indicates that the mammary repopulating units with functional stem cell activity are not contained within the SP [228]. Hence the SP technique is no longer the preferred approach for stem cell studies.

3.11.2. Expression of cell surface markers

A number of studies have demonstrated that expression of stem-cell markers in mammary tumors has prognostic significance [46]**. T**he choice of marker can greatly vary depending on tissues or species. The following markers have been used in the study of breast stem cells (Figure. 3.12).

3.11.2.1. CD44+/CD24-/low lin-

Clarke and colleagues demonstrated that as few as 200 of CD44+/CD24- /low/lin- cells generated tumors in NOD/SCID mice whereas 20,000 cells that did not display this phenotype failed to do so. The NOD/SCID tumors recapitulated the entire heterogeneity of the initial tumor. These cells were able to self-renew, to differentiate, and displayed tumorigenic capacity. However, its application is limited by the great cellular heterogeneity of the CD44+/CD24-/low/ lin- population, which probably does not contain solely *bona fide* CSCs. The use of the CD44+/CD24-/low/lin- phenotype and another marker, the ALDEFLUOR assay, which measures the Aldehyde dehydrogenase enzymatic activity, demonstrated that cells able to initiate tumor in mice were within the ALDEFLUOR-positive cells, the cells displaying both phenotypes being the most tumorigenic, and that none of the CD44+/CD24-/low/lin- cells without ALDEFLUOR activity could grow in mice [26]. These results indicate that the CD44+/CD24-/low/lin- population contains some but not all the CSCs in breast tumors [28, 232].

3.11.2.2. CD49f/ITGA6/α6-integrin

Cells expressing CD49f/ITGA6/α6-integrin surface markers represent a rare subset of adult mouse mammary stem cells that were able individually to regenerate an entire mammary gland within six weeks *in vivo* [233]. Interestingly, a recent study described a subpopulation that overexpressed the α6-integrin in the human breast

cancer cell line MCF7. This population is capable of forming spherical organoids in anchorage-independent conditions, resistant to pro-apoptotic agents and can form tumors in immunodeficient mice [234].

3.11.2.3. CD133

CD133 might be a good candidate to explore carcinogenesis and estrogen-dependent tumor progression. In cell lines from mammary tumors *Brca1* knockout mice, the expression of CD133 cells is associated with stem cell properties [235].

3.11.2.4. CD29/β1-integrin and CD61/β3-integrin

In the mouse model, mammary stem cells are enriched using the CD29high/CD24+ and $CD61^+$ markers [236] [237]. CD29 and CD61 could therefore be promising targets for human clinical applications based on data obtained from normal and tumoral mouse mammary gland studies.

3.11.2.5. ALDEFLUOR assay

The ALDEFLUOR assay may fit the universality required for a stem cell marker to be reliable across species and tissues. It is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid. ALDH1 may have a role in early differentiation of stem cells [238, 239]. High ALDH1 activity is associated with several types of murine and human stem hematopoietic and neural stem and progenitor cells [240, 241]. ALDEFLUOR- positive cells isolated from both normal and tumoral human breast had phenotypic and functional characteristics of mammary stem cells. Furthermore, the ALDEFLUOR-positive population isolated from human breast tumors contained the CSC population as demonstrated by the ability of these cells but not of ALDEFLUOR- negative cells to generate tumors in NOD/SCID mice. Serial passages of ALDEFLUOR-positive cells generated tumors recapitulating the phenotypical diversity of the initial tumor [26]. However, the ALDEFLUOR assay does have some limitations for the isolation of tumorigenic population in tumors of different origin. For example, both ALDEFLUOR [66] and ALDEFLUOR (low) from the lung carcinoma cell line H 522 were able to initiate tumors after inoculation into NOD/SCID mice.

3.11.2.6. In situ detection

In situ detection of stem cells has the potential to transfer stem cell quantification to routine clinical practice for patient treatment and prognosis evaluation.

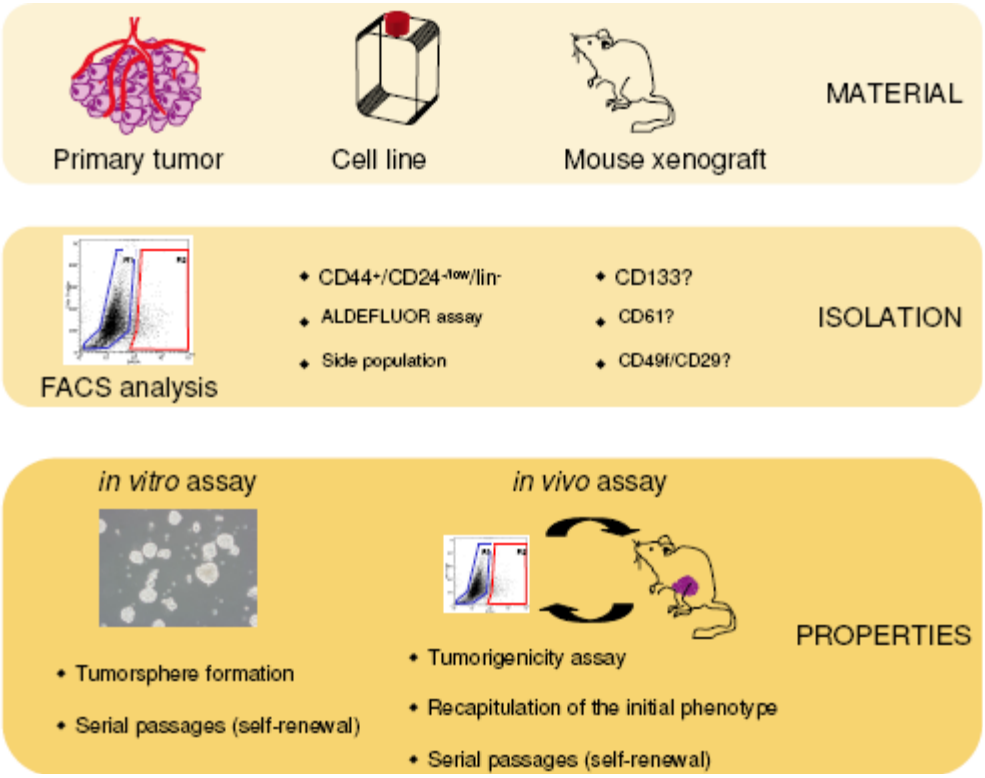
Aldehyde dehydrogenase activity has been mostly attributed to the function of aldehyde dehydrogenase1A1, one of the main ALDH cytoplasmic isoform [238]. *In situ* immunostaining of ALDH1A1 has been measured in formalin-fixed, paraffin embedded breast tumors and it identified both normal and malignant human mammary stem cells; 30% of the breast tumors analyzed presented a relatively small ALDH1-positive cell population. The analysis of ALDH1 expression in human breast carcinomas showed that the expression of this stem/progenitor cell marker is a powerful predictor of poor clinical outcome [26].

Another method used to detect stem cells *in situ* is double immunostaining using CD44 and CD24 antibodies and subsequent quantification of the CD44+/CD24low/- phenotype. The prevalence of CD44+/CD24low/- cells in paraffin- embedded tumors was lower than 10% in the majority of cases, and this phenotype was neither associated with clinico-pathologic characteristics nor with clinical outcome [242]. Also, the CD44+/CD24low/- phenotype was surprisingly scarce in ERBB2-positive tumors, which had a predominantly CD24+ status [243]. Even if the CD44+/CD24low/- phenotype is a valuable marker for the isolation of breast CSCs, because this stem cell component within primary tumors can be scarce and is mainly associated, with basal like/ BRCA tumors only, it cannot be used in clinical settings [244].

3.11.2.7. Anchorage-independent cell culture

Human mammary stem and progenitor cells were able to survive in suspension and produce spherical colonies (mammospheres) composed of both stem and progenitor cells. These non-adherent mammospheres were enriched in early progenitor/stem cells and able to differentiate along the three mammary epithelial lineages and to clonally generate complex functional structures in reconstituted 3D culture systems as well as reconstitute human normal mammary gland in mice [44].

The mammosphere assay, is used to establish long term cultures enriched in stem/progenitor cells from invasive tumor samples. They showed an increase in SP fraction and CD44+/CD24-//low cells, overexpressed neo-angiogenic and cytoprotective factors, expressed the putative stem cell marker OCT4, and displayed high tumorigenic potential in NOD/SCID mice [245]. Thus, the development of *in vitro* suspension culture systems not only provides an important new tool for the study of mammary cell biology, but also has important implications for understanding key molecular pathways in both normal and neoplastic stem cells.



Adapted from BMC Cancer, 2009

Figure 3.12 Markers and model for breast cancer stem cell studies. The main assays, markers and models used to study breast cancer stem cells are schematically represented. Models and assays rely on the main stem cells properties that are self-renewal ability and differentiation potential. The various markers illustrate the great phenotypic diversity of the cancer stem cell population.

3.11.3. Resources for the characterization of breast cancer stem cells

3.11.3.1. Breast cancer cell lines

CSCs have been isolated from different mouse and human breast cancer cell lines. A subpopulation of MCF7 was able to grow as spherical organoids in anchorage-independent conditions, displayed resistance to pro-apoptotic agents and greater tumorigenicity than its parental line in immunodeficient mice [234]. Several other studies have used putative CSC markers such as CD44+/CD24-/low to identify similar populations within breast cancer cell lines, but given that CD44 is a basal marker, this phenotype did not isolate the tumorigenic population [246]. Wicha et. al., used the ALDEFLUOR assay to isolate and characterize CSCs from 33 breast cell lines, derived from normal and malignant mammary tissue [247]. 23 of the cell lines contained an ALDEFLUOR-positive population that displayed stem cell properties *in vitro* and in NOD/SCID xenografts. The use of cell lines can facilitate the characterization of regulatory pathways of cancer stem cells and identify potential stem cell markers and therapeutic targets.

3.11.3.2. Human xenograft models

Despite the caveats represented by a change in the functional properties of CSCs in the animal host and the changes in the niche (tumoral stroma, hormonal influence), the xenograft model of patient samples appears to be the closest experimental system to tumors in human patients [248]. Vasculature, stroma, central necrosis, and peripheral growth occur in tumor-bearing mice in a way that is similar to that of the patient's tumor. Furthermore, tumor xenografts are the most relevant way to test CSC properties such as the ability to form tumors, self-renewal potential and capacity to differentiate. Among the large variety of tumors transplantable into immunodeficient mice, breast cancers are among the most difficult to establish [249].

3.11.4. Metastasis and the stem-cell phenotype

There is increasing evidence that cancer stem cells play an important role in mediating tumor metastasis. Aldefluor-positive populations of mammary carcinoma cell lines display increased invasive characteristics as well as increased ability to metastasize when injected into the left ventricle of NOD/SCID mice

[250]. There is increasing evidence that the tumor microenvironment plays an important role in tumor growth and metastasis.

3.11.5. Cancer treatment in the wake of cancer stem cells

Breast cancer stem cells [22], as well as cancer stem cells from other tumor types, are relatively resistant to both radiation and chemotherapy [251]. Current clinical trial designs are largely been based on strategies aimed at producing tumor regression. However, in many malignancies including breast cancer, tumor regression does not correlate well with patient survival [252]. Limitations of present therapies may relate to their inability to target the cancer stem cell component. Recent neoadjuvant studies demonstrating an increase in the proportion of CD44+/CD24− and ALDH-positive breast cancer stem cells after chemotherapy suggest that this is the case [253, 254]. The elucidation of pathways that regulate breast cancer stem cells, such as Notch, Hedgehog, and Wnt, provide new targets for therapeutic development. The ultimate test of this hypothesis, however, will be the demonstration that the successful targeting of cancer stem cells results in improved clinical outcomes for patients with breast cancer.

3.12. Computational Biology

Biological systems are driven by interactions between biomolecules-DNA, proteins and ligands. Protein-protein/ligand interactions are involved in virtually all the cellular processes including metabolism, signalling and development. Given the increased focus on interactions in the current post-genomics era, structural knowledge of complexes is required to understand how the various biomolecular units work together to fulfil their tasks. Computational approaches to predict protein binding site and protein-protein complex structure and protein-ligand interactions are powerful tools to gain such structural knowledge and improve our understanding of protein function and their recognition mechanisms. Protein-ligand interactions can be predicted and studied by performing homology modeling for the structural prediction of the target protein, molecular dynamic simulation studies of the predicted homology model and docking of ligands with the target protein.

Initial modeling studies were based on the construction of wire/plastic models followed by the usage of interactive computer graphics. Browne *et al.* [255] modeled bovine α- lactalbumin on the known 3D structure of hen egg white lysozyme. Later on Warme *et al.* [256] produced a model for α- lactalbumin on the basis of the crystal structure of lysozyme. Both of these models were constructed by taking the existing coordinates of the known structure, and mutating side chains not identical in the protein to be modeled. This approach to protein modeling is still employed today with considerable success, especially when the proteins are similar.

3.12.1. Homology or Comparative Modeling

The most reliable technique for predicting protein structures is homology modeling, provided the geometry of one or more template proteins with sufficient sequence identity are given. If the sequence identity between template and target protein is high enough, the resulting model may even be sufficiently accurate to perform structure-based drug design. Building a model by homology requires a general approach leading from the selection of a suitable template structure and its alignment with the target sequence to the refinement of the full model. Rules can be deduced from existing structures to guide and improve the modeling process. A flow diagram of the modeling process is shown in Figure 3.13.

The first step in homology modeling is to scan a structural database for suitable template structures. This can be done with sequence comparisons or more sophisticated fold recognition methods. Finding a significant sequence similarity implies inferring a plausible correspondence between residues of both sequences. Selection of the template structures is therefore generally combined with aligning the template sequence to the target. For higher sequence identities (≥ 45%) this is straightforward. Most programs will roughly produce a similar alignment. The computation of an alignment becomes extremely difficult for very low identity (<= 25%). The final steps of the model building process consist in assessing the quality of the model and adjusting small errors. A limited energy minimization of the structure may also be employed to reduce local clashes between atoms. The stereochemical quality may be assessed, which indicate deviations from ideal

bond lengths and angles. Energy functions are useful to both estimate the overall quality and to perform energy minimization.

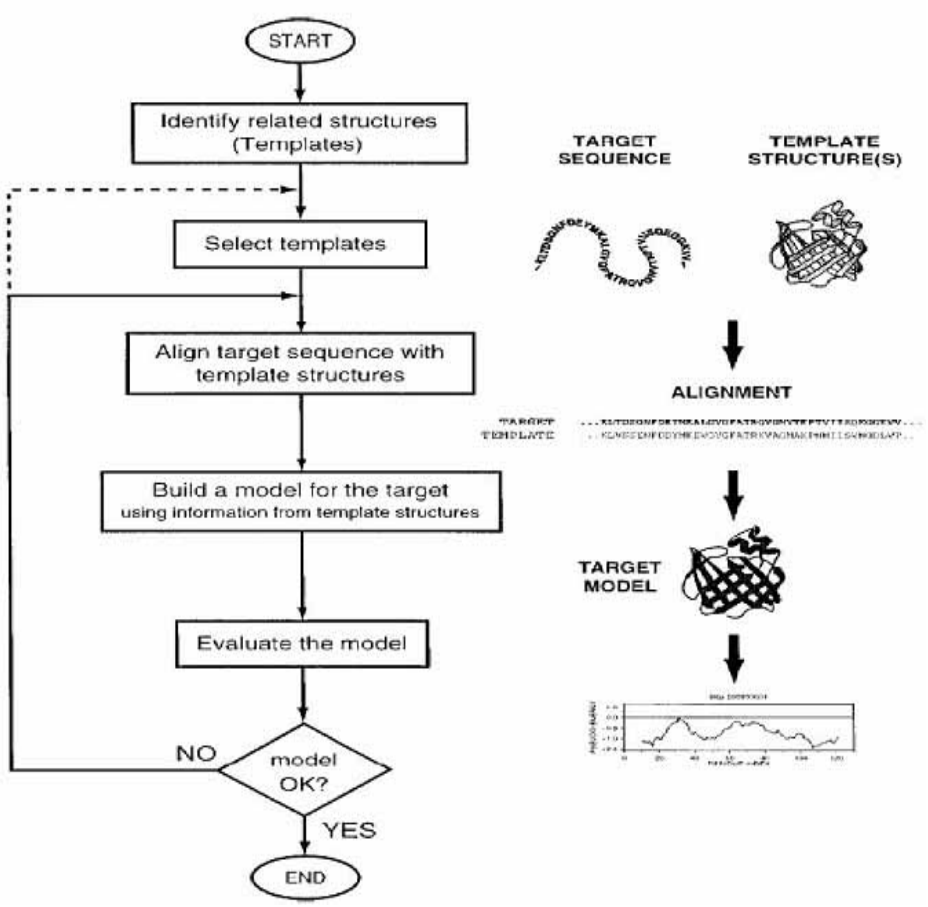


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Figure 3.13 Sequential steps of homology modeling for prediction of 3-D structure of proteins.

Although considerable progress has been made in physical structure prediction methods and combinatorial modeling, homology modeling techniques remain the only modeling method that produce models with rms error lower than 2 A for sequences that have sufficient similar homologues with known 3D structure [257]. In contrast, physical prediction methods and combinatorial modeling calculate structure with rms errors of approximately 3.5 A for small proteins [258]. Homology modeling is not as accurate as X-ray crystallography and NMR, which can determined protein structure with an rms error of approximately 0.3 and 0.5 A respectively [259]. Though this technique is restricted to sequences with closely

related proteins with known 3D structures, it can provide starting models for X-ray crystallography and NMR spectroscopy. Various software and online tools were used in building a homology model.

3.12.1.1. Software Components:

1. MODELLER is a computer program used in producing homology models of protein tertiary structures as well as quaternary structures which implements a technique inspired by nuclear magnetic resonance known as *satisfaction of spatial restraints*, by which a set of geometrical criteria are used to create a probability density function for the location of each atom in the protein. The method relies on an input sequence alignment between the target amino acid sequence to be modeled and a template protein whose structure has been solved. MODELLER was originally written and is currently maintained by Andrej Sali at the University of California, San Francisco.

2. PyMOL: PyMOL is a visualization tools used in structural biology. The Py portion of the software's name refers to the fact that it extends, and is extensible by the Python programming language. It can produce high quality 3D images of small molecules and biological macromolecules, such as proteins, DNA etc. Almost a quarter of all published images of 3D protein structures in the scientific literature were made using PyMOL. (http://www.delanoscientific.com/)

3. Visual molecular dynamics (**VMD**) : VMD is a molecular modelling and visualization computer program which is primarily developed as a tool for viewing and analyzing the results of molecular dynamics simulations, but it also includes tools for working with volumetric data, sequence data, and arbitrary graphics objects. Molecular scenes can be exported to external rendering tools such as POV-Ray, Renderman, Tachyon, VRML, and many others. (http://www.ks.uiuc.edu/Research/vmd/)

4. Clustal is a widely used multiple sequence alignment computer program. The latest version is 1.83. There are two main variations:
Clustal-W: command line interface

Clustal-X: This version has a graphical user interface. It is available for Windows, Mac OS and Unix/Linux

3.12.1.2. On line Tools

In addition to the above software, various on line computational tools used in the present study were as denoted below.

1. National Center for Biotechnology Information (NCBI): The NCBI is part of the United States National Library of Medicine (NLM). The NCBI houses genome sequencing data in GenBank and an index of biomedical research articles in PubMed Central and PubMed, as well as other information relevant to biotechnology. All these databases are available online through the Entrez search engine. (www.ncbi.nlm.nih.gov). Popular resources that are present at NCBI are BLAST (Basic Local Alignment Search Tool), Bookshelf, Gene, Genome, Nucleotide, OMIM (Online Mendelian Inheritance in Man), Protein, PubChem, PubMed, PubMed Central, SNP (Single Nucleotide Proteins) (http://www.ncbi.nlm.nih.gov).

2. PDB : The **Protein Data Bank** (**PDB**) PDB is a repository for the 3D structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are freely accessible on the internet via the websites of its member organizations.

3. Pfam : Pfam is a collection of protein motifs and families maintained by the Bioinformatics group at the Sanger Centre. Pfam hidden Markov models (HMMs) and the Prosite generalized profiles were developed based on distinct theoretical backgrounds. (http://www.sanger.ac.uk/Software/Pfam/ftp.shtml).

4. SWISS-Prot: This is a manually curetted biological database of protein sequences created in 1986 by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute. Swiss-Prot strives to provide reliable protein sequences associated with a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications,

variants, etc.), a minimal level of redundancy and high level of integration with other databases. (http://expasy.org/sprot/). This program is available from European Bioinformatics Institute ftp server.

5. Superpose: SuperPose is a protein superposition server which calculates using a modified quaternion approach. The SuperPose generates sequence alignments, structure alignments, PDB coordinates, RMSD statistics, Difference Distance Plots, and interactive images of the superimposed structures. The SuperPose web server supports the submission of either PDB-formatted files or PDB accession numbers.

6. PDBSUM : The PDBsum is a pictorial database that provides an at-a-glance overview of the contents of each 3D structure deposited in the Protein Data Bank (**PDB**). (http://www.ebi.ac.uk/pdbsum/)

7. PRODRG : The PRODRG will convert coordinates of small molecules in PDB format to the topology formats of GROMACS, GROMOS, WHAT IF, AUTODOCK etc. In addition coordinates for hydrogen atoms are generated.

8. ProSA-web: ProSA program which exploits the advantages of interactive web-based applications for the display of scores and energy plots that highlight potential problems spotted in protein structures. In particular, the quality scores of a protein are displayed in the context of all known protein structures and problematic parts of a structure are shown and highlighted in a 3D molecule viewer. The service specifically addresses the needs encountered in the validation of protein structures obtained from X-ray analysis, NMR spectroscopy and theoretical calculations.

9. Sequence analysis : Various types sequence analysis were carried out through retrieving the sequences either from NCBI or SWISS-Prot databases. Sequence homology search was conducted through the BLAST-p program available at NCBI. Homology modeling of target sequence needs a template crystal structure coordinates which were obtained by performing BLAST-p at NCBI with selection of database as PDB. The coordinates of selected crystal structures of sequence

similar structures of target protein were obtained from PDB and used for prediction of 3-D structure of target protein using MODELLER. In order to identify conserved and variable regions of the sequences and in determining the most robsi gap arrangement, multiple sequence alignment of all homologous proteins of the target sequence Clustal-W with appropriate parameters were used as per the specified instructions.

10. PDBsum: PDBsum is database that provides an overview of the contents of each 3D macromolecular structure deposited in the Protein Data Bank. Each structure in the PDBsum database includes an image of structure, molecular components contained in the complex, enzyme reaction diagram if appropriate, Gene Ontology functional assignments, a 1D sequence annotated by Pfam and InterPro domain assignments, description of bound molecules and graphic showing interactions between protein and secondary structure, schematic diagrams of protein-protein interactions, analysis of clefts contained within the structure and links to external databases.

3.12.2. Docking

Docking techniques, designed to find the correct conformation of a ligand and its receptor, have now been used for decades. The process of binding a small molecule to its protein target is not simple; several entropic and enthalpic factors influence the interactions between them. The mobility of both ligand and receptor, the effect of the protein environment on the charge distribution over the ligand [260], and their interactions with the surrounding water molecules, further complicate the quantitative description of the process. The idea behind this technique is to generate a comprehensive set of conformations of the receptor complex, and then to rank them according to their stability. The most popular docking programs include DOCK, AutoDock, FlexX, GOLD, and GLIDE among others.

3.12.2.1. AutoDockTools (ADT): ADT is the GUI for AutoDock to set up, launch and analyze AutoDock runs. With ADT one can view molecules in 3D, rotate and scale in real time, add all hydrogens or just non-polar hydrogens, assign partial atomic charges to the lignad and macromolecule, merge non-polar hydrogens and their charges with their parent carbon atom, set up rotatable bonds in the ligand

using graphical version of Autotors, set up the Autogrid Parameter File (GPF) using a visual representation of the grid box, and slider-based widjets, set up the AutoDock parameter File (DPF) using forms, read in the results of an AutoDock job and graphically display them.

3.12.2.1.2. AutoDock: AutoDock is molecular modeling simulation software effective for Protein-ligand docking that includes how small molecules, such as substrates or drug candidates bind to receptor of known 3D structure. AutoDock consists of two main programs: AutoDock performs the docking of the ligand to a set of grids describing the target protein; AutoGrid for pre-calculating these grids. In addition to using them for docking, the atomic affinity grids can be visualized. This can help, for example, to guide organic synthetic chemists design better binders.

3.12.2.2. Molinspiration: Molinspiration is a cheminformatics software tool, supporting molecule manipulation and processing, including SMILES and SDfile conversion, normalization of molecules, generation of tautomers, molecule fragmentation, calculation of various properties needed in QSAR, drug design and high quality molecule design.

3.12.3. Molecular Dynamics (MD) Simulations

Molecular dynamics simulations are one of the most versatile and widely applied computational techniques for the study of biological macromolecules [261, 262]. They are very valuable for understanding the dynamic behavior of proteins at different timescales, from fast internal motions to slow conformational changes or even protein folding processes. It is also possible to study the effect of explicit solvent molecules on protein structure and stability to obtain timeaveraged properties of the biomolecular system, such as density, conductivity, and dipolar moment, as well as different thermodynamic parameters, including interactions energies and entropies.MD is useful not only for rationalizing experimentally measured properties at the molecular level, but it is well known that most structures determined by X-ray or NMR methods have been refined using MD methods. Therefore, the interplay between computational and experimental techniques in the area of MD simulations is longstanding, with the theoretical

methods assisting in understanding and analyzing, experimental data. These, in turn, are vital for the validation and improvement of computational techniques and protocols. Although the first protein MD simulation was done in-vaccuo and for only 8.8 psec, enormous increases in computer power now a days permit simulations of systems comprising 104.106 atoms [263, 264] and simulation times in the order of nsec to msec [265]. Simulations of more realistic systems, including explicit water molecules, counter ions, and even a complete membrane-like environment are possible, and new properties can now be studied as they evolve in real time.

This progress in system representation has been accompanied by methodological improvements: better force fields, improved treatment of long-range electrostatic interactions and system boundary conditions, and better algorithms used to control temperature and pressure. However, despite all these advances, the set up of an MD simulation can be far from trivial. Parameters used to describe proteins and their interactions are normally found within modern force fields, but adequate descriptors for non-standard molecules, such as ligands, might be missing. In such cases, the determination and fitting of new parameters is usually straightforward, but may be a time-consuming process if it needs to be done for many ligands, limiting the general applicability of the method. Commonly used programs for MD simulations of biomolecules include Amber, CHARMM, GROMOS, and NAMD among others. Fast and inexpensive docking protocols can be combined with accurate but more costly MD techniques to predict more reliable protein.ligand complexes. The strength of this combination lies in their complementary strengths and weaknesses. On the one hand, docking techniques are used to explore the vast conformational space of ligands in a short time, allowing the scrutiny of large libraries of drug-like compounds at a reasonable cost. The major drawbacks are the lack or poor flexibility of the protein, which is not permitted to adjust its conformation upon ligand binding, and the absence of a unique and widely applicable scoring function, necessary to generate a reliable ranking of the final complexes. On the other hand, MD simulations can treat both ligand and protein in a flexible way, allowing for an induced fit of the receptor-binding site around the newly introduced ligand. In addition, the effect of explicit water molecules can be

studied directly, and very accurate binding free energies can be obtained. However, the main problems with MD simulations are that they are time-consuming and that the system can get trapped in local minima. Therefore, the combination of the two techniques in a protocol where docking is used for the fast screening of large libraries and MD simulations are then applied to explore conformations of the protein receptor, optimize the structures of the final complexes, and calculate accurate energies, is a logical approach to improving the drug-design process.

3.12.3.1. Gromacs (Groningen Machine for Chemical simulations) is a molecular dynamics simulation package originally developed in the University of Groningen. The program is written for Unix-like operating system; it can run on Windows machines if the cygwin Unix layer is used. Gromacs contains a script to convert molecular coordinates from a PDB file into the formats it uses internally. Once a configuration file for the simulation has been created, the actual simulation run produces a trajectory file, describing the movements of atoms over time. This trajectory file can be analyzed or visualized with a number of supplied tools.

3.12.3.2. VMD: Visual molecular dynamics (VMD) is a molecular modelling and visualization computer program. VMD is primarily developed as a tool for viewing and analyzing the results of molecular dynamics simulations, but it also includes tools for working with volumetric data, sequence data, and arbitrary graphics objects. Molecular scenes can be exported to external rendering tools such as POV-Ray, Renderman, Tachyon, VRML, and many others. Users can run their own Tcl and Python scripts within VMD as it includes embedded Tcl and Python interpreters.

Chapter 4

Establishment and Characterization of Breast Cancer Cell Lines from Primary Breast Tumors in Indian Women

Chapter 4: Establishment and Characterization of Breast Cancer Cell Lines from Primary Tumors in Indian Women

4.1. Introduction:

Breast cancer is the leading cause of cancer deaths among women, accounting for 23% of the total cancer incidence and 14% cancer deaths globally [266]. In India breast cancer has emerged as most common cancer in women, which was earlier reported as second most common cancer after cancer of cervix [65], the age adjusted annual incidence rate (AAR) ranging from 25-33 cases per 100,000 women in urban population and 7.2 in rural areas [65]. Around 100,000 women are diagnosed with carcinoma breast every year in India, of which around 50,000 women die with the disease every year with a predicted rise to 131 000 cases by 2020, and increased concentration in urban areas [267]. The incidence of breast cancer in Indian population (1/35) is not as high as in the western countries (1/8) however, the incidence of early onset of breast cancer cases (<40 years) does not show significant variation in women worldwide (12-33 per 100,000 women); suggesting that a greater proportion of all breast cancers is mainly due to early onset of disease in Indian population [268]. The average age of onset of breast cancer in Indian patients ranges between 40-50 years compared to 60-70 in western countries. Breast cancer diagnosed at young age is well recognized as clinically different than breast cancers diagnosed at older ages [269]. Younger patients more frequently exhibit aggressive features such as large tumor size, high histological grade, positive lymph nodes, absence of steroid receptors and high S-phase fraction, and young age itself has been shown to be an independent predictor of adverse prognosis [270-273]. Majority of Indian breast cancer patients are young (<40yrs), married and having children at younger age with history of breast feeding their children for long duration. The development of breast cancer in these young women is an enigma. There is not much information available regarding the molecular mechanism and etiological factors responsible for the breast cancer at young age; hence there is a need for establishing an experimental tool to investigate it.

Cell lines provide an important experimental tool in cancer research with major benefit of infinite supply of a relatively homogeneous cell population that is capable of self-replication which can be widely distributed to facilitate comparative studies. Cell cultures established directly from human tumors serve as unique models for studying and manipulating the potentially relevant molecular and cellular processes underlying malignant disease and identification of novel biological therapeutic targets. Majority of breast cancer derived cell lines are from secondary tumours and pleural effusions of patients with advanced stage breast cancers [13-15, 113, 114, 117, 118, 121, 274]. Few breast cancer cell lines have been successfully established from primary tumours [16-18, 119, 120, 275]. Among the available breast cancer cell lines, majority of cell lines are established from cancers from older age group patients (> 55yrs), only a small proportion of breast cancer cell lines are established from patients <40 years of age. To establish a tool to elucidate the molecular pathogenesis of breast cancer in young Indian women who usually exhibit major reproductive protective factors for breast cancer, we have established two breast cancer cell lines, NIPBC-1 and NIPBC-2 from primary tumours of two young breast cancer patients (<40yrs). These two cell lines are triple negative which is most common phenotype seen in breast tumours in young women.

4.2. Materials and methods:

4.2.1. Establishment and purification of primary cultures:

Primary cultures were established from tumour tissue obtained from breast cancer patients, who underwent modified radical mastectomy (MRM) or trucut biopsies at Safdarjung hospital, New Delhi, India. A part of biopsy was used for frozen section to confirm the diagnosis and presence of tumor cells in it. The tumour tissue was collected in DMEM supplemented with antibiotics (penicillin 100U/ml, streptomycin 100µg/ml). Tissue were minced into 1-2 cu.mm pieces using a cutting instrument, such as scalpel blade to remove blood, fat and fibro connective tissue and transferred into tissue culture flask containing DMEM supplemented with 10-20% FBS (fetal bovine serum), Glutamine for example 2 mM and growth factors such as epidermal growth factor (5-15 ng/ml) for enzymatic disaggregation.

Initially, only limited success was achieved due to several inherent problems in obtaining contamination free breast tissue cultures. The most perpetual problem encountered during the establishment of primary cultures/cell lines from excisional biopsies or core biopsies were frequent bacterial/fungal contamination and deprivation of tumor tissue. Chronic exposure of the external breast lesions to unhygienic ways of cleaning, increased the probability of contamination. Some of the other problems included, low yield of viable cells, very low yield of epithelial cells, poor adherence to the cell culture flask, slow growth, cell division and outgrowth of fibroblasts. To circumvent these problems several strategies were tried:

a) Tissue specimens were washed several times in DMEM containing solution of antibiotics (streptomycin, 100 ug/ml; pencillin 100 U/ml) and fungizone (0.25 ug/ml);

b) Tissue specimens were treated with different concentrations of collagenase or dispase to improve the yield of epithelial cells;

c) Different combinations of enzyme concentrations were tried to reduce the tissue digestion time, so as to increase the cell viability;

d) Adherence of the cells was improved by testing several solid support systems such as precoating the tissue culture flask with varying concentrations of polylysine or collagen;

e) To combat the problem of slow cell division various strategies were tried such as high FBS concentration, supplementation of growth medium with different growth factors such as Insulin transferrin-selenium alone or in combination and epidermal growth factor (EGF, 10 ng/ml), hydrocortisone and estrogen.

Enzymatic disaggregation was carried out by incubating the small tissue pieces with 2.5% crude trypsin for 30 minutes at 37°C and with collagenase (0.15%) overnight. Cells released after enzymatic treatment were tested for cell viability using trypan blue and then seeded on to a tissue culture flasks and maintained in DMEM medium supplemented with 10-20% FBS, Glutamine 2mM, and growth factors such as epidermal growth factor (5-15 ng/ml) insulin 100U/ml. The cells

were then maintained in DMEM medium supplemented with 10% FBS and growth factors at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Using several different permutations/combinations and different concentrations of EGF, we could finally overcome these problems encountered during the process of establishment of primary cultures from breast cancer tissue biopsies.

One of the major problems faced in cultivation of epithelial cells is the out growth of fibroblasts. Fibroblasts grow more rapidly than epithelial cells and hence made culturing of epithelial cells quite difficult. Primary epithelial cultures that contained fibroblasts were treated with increasing concentrations of trypsin-EDTA (Differential trypsinisation method). Alternatively they are incubated with geneticin to kill the fibroblast cells. Further the primary cultures are purified by Magnetic activated cell sorting using EpCAM (Epithelial cell adhesion molecule) and Cytokeratin antibodies; by isolating a single clone and growing them in 96-well plates (Dilution cloning method). Primary cultures were treated with different permutations and combinations of the above methods to obtain a predominant population of epithelial cells, essentially free of fibroblasts. These cultures were allowed to grow to sub-confluency. Thereafter, the subconfluent cultures were subcultured and passaged at periodic intervals. Growth factors were gradually withdrawn from the primary cultures after their purification and were maintained in DMEM medium supplemented with 10% FBS and 2mM Glutamine. Once the cultures were 80- 90% confluent, the cells were trypsinised with 0.05% trypsin and split in a ratio of 1:3 in fresh DMEM medium. Also, the cells were sampled and frozen periodically at various passages.

During the course of this study, 44 breast tumours were collected and used for initiation of primary cultures (Table 4.1). Different cultures required 5-15 days to become confluent (Figure 4.1). Of the total samples collected, 23 were from early onset (<45 years) and 21 are from late onset (>50 years) breast cancers (Table 4.1.). Together we established 21 primary cultures. Among the above cultures, NIPBC-1 and NIPBC-2 were completely purified, passaged for 85 times and 66 times respectively and were characterised thoroughly hence considered as established continuous cell lines.

4.2.2. Characterisation of established cell lines:

4.2.2.1. Phenotypic Characterization:

(i) Immunoflourescence:

For immonocytochemical analysis cells were grown on cover slips, fixed with methanol-acetone/ 4% paraformaldehyde and incubated with monoclonal antibodies for Estrogen receptor (ER), Progesterone receptor (PR), HER2/neu, Pan-Cytokeratin, Cytokeratins 5/6 and 18, vimentin, epithelial membrane antigen (EMA), Mucin 1 and P53. These cover slips were further incubated with secondary antibody conjugated with FITC (DAKO) and counterstained with propidium iodide (Sigma). Fluorescence was detected using fluorescence microscope.

Patient ID	Age	TNM	Family History	Histopathology Report
PCB1	50/F	T2N3M0	No	IDC
PCB2	50/F	TxNxMx	No	Benign lump
PCB3	40/F		No	Benign lump
PCB4	55/F	T4aN2M0	No	IDC
PCB5	45/F	T4bN1M0	No	IDC
PCB6	35/F	T4bN2M0	Yes	IDC
PCB7	56/F	T4aN0M0	No	IDC
PCB8	45/F		Yes	IDC
Patient ID	Age	TNM	Family History	Histopathology Report
PCB9	31/F	T4aN0M1	No	IDC
PCB10	42/F	T4bN2M0	No	IDC

PCB11	**35/F**		**No**	**IDC**
PCB12	**50/F**	**T4N2M0**	**No**	**IDC**
PCB13	**38/F**	**T3N0M0**	**No**	**IDC**
PCB14	**87/F**		**No**	**IDC**
PCB15	**40/F**	**T3N0M0**	**No**	**IDC**
PCB-16	**42/F**	**T4bN1M0**	**No**	**IDC**
PCB-17	**42/F**	**T2/3N1M0**	**No**	**IDC**
PCB-18	**50/F**		**No**	**DCIS**
PCB-20#	**39/F**	**T2/3N1M0**	**No**	**IDC**
PCB- 22	**70/F**		**No**	**IDC**
PCB- 23	**40/F**	**T4bN1M0**	**No**	**IDC**
PCB- 24	**60/F**	**T4dN2M0**	**No**	**IDC**
PCB-25	**42/F**	**T3N2M0**	**No**	**IDC**
PCB- 26	**37/F**	**T2N0M0**	**No**	**IDC**
PCB- 27	**52/F**	**T4cN2M0**	**No**	**IDC**
PCB- 28	**45/F**		**No**	**IDC**
PCB-29	**42/F**	**T1N1M0**	**No**	**IDC**
PCB-30	**38/F**	**T2N0M0**	**No**	**IDC**
PCB-31	**52/F**	**T2N1M0**	**No**	**IDC**
PCB-32	**53/F**	**T2N1M0**	**No**	**IDC**
PCB-33	**40/F**	**T2N2M0**	**No**	**IDC**

Patient ID	Age	TNM	Family History	Histopathology Report
PCB-34	40/F	T2N1M0	No	IDC
PCB-35	44/F	T2N1M0	No	IDC
PCB-36#	38/F	T2N1M0	No	IDC
PCB-37	25/F	T4N2M2	Yes	IDC
PCB-38	64/F	T4N1M0	No	IDC
PCB-39	55/F	TxN3M0	No	IDC
PCB-40	45/F	T4N2M0	No	IDC
PCB-41	38/F		No	IDC
PCB-42	40/F	T2N0M0	Yes	IDC
PCB-43	35/F	T4BN1M0	Yes	IDC
PCB-44	42/F	T1N1M0	No	IDC

Table 4.1. Clinico-pathological details of the samples collected

 # Cell cultures developed into cell lines and named as NIPBC-1 and NIPBC-2 respectively

IDC- Infiltrating Ductal Carcinoma

DCIS- Ductal Carcinoma InSitu

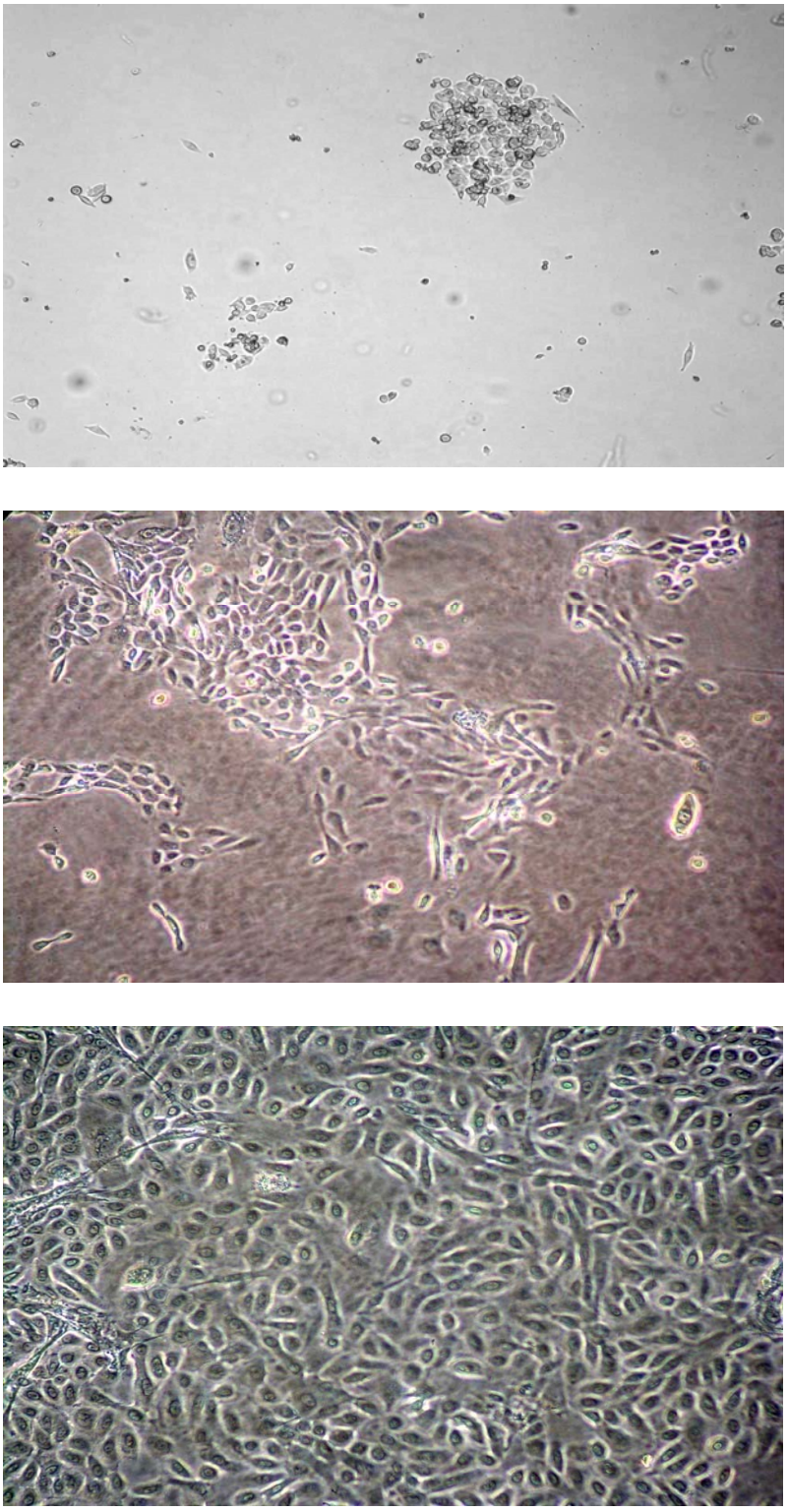

Figure 4.1. Representative pictures of PCB34 culture showing (a) Initiation at day 5 (b) 5 days after partial purification by enzymatic treatment (c) 10 days after partial purification.

(ii) Flowcytometry:

Cells were trypsinised to form a single cell suspension, counted and washed with ice cold staining buffer (1x PBS with 3% FBS and 0.05% sodium azide). Approximately, 1x 10^6 cells were resuspended in 500 µl of ice cold staining buffer and 3µl of FITC conjugated Ki67 or primary antibodies of p53 were added and incubated for 30 minutes at 4°C in dark. The cells were then washed and incubated subsequently with FITC conjugated secondary antibodies (for P53 and P21). After incubation the cells were washed twice by centrifugation at 400g for 5 minutes and resuspended in 1ml of ice cold staining buffer and kept on ice until analysis. Appropriate isotype controls have been used.

4.2.2.2. Ultrastructure analysis of the cells:

For ultrastructure studies, cultured cells were double fixed with 1.2% glutaraldehyde in 0.01 M phosphate-buffered saline (pH 7.5) and with 2.0% glutaraldehyde for l hr at 4°C. Post-fixation was performed with 1% OsO4. The sample was embedded in epoxy resin. The ultra- thin sections were cut (400-500µ), stained by lead citrate and uranyl acetate and studied under transmission microscope [276].

4.2.2.3. Softagar assay:

The ability of anchorage-independent growth of the purified cancer epithelial cells was determined by growing the cells on semisolid agar at 25th and at 40th passages. A single-cell suspension containing 10^5 cells/35-mm petri dish was dispersed in a solution containing 0.3% bacto-agar in epithelial culture medium described above. This was then layered over a 0.6% bacto-agar solution in DMEM/F12 supplemented with 10% FBS and fed biweekly with epithelial cell culture medium. Formation of colonies was determined by inverted microscopy for 2-3 weeks post-seeding [277].

4.2.2.4. Karyotyping:

Karyotyping of both NIPBC-1 and NIPBC-2 cell lines has been done at both early passages (P20 and P15 respectively) and late passages (P65 and P52 respectively)

to facilitate cytogenetic examination and comparison of the karyotypes between early and late passages. Briefly, cells were plated at approximately 1-2x 10^6/75 cm^2 flask. After 48 hrs cells were exposed to 0.1μg of colchicine When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells (data not shown). Thus, we hypothesize that lack of mammosphere formation could not be attributed to the absence of live cells beyond the sixth/seventh passage, but due to differentiation of cancer stem cells.

Sigma) at 37^0C, for 3 hrs. The cells were then harvested by trypsinization, incubated for 20 minutes at room temperature with a hypotonic solution (75mM KCL) and fixed with methanol: acetic acid (3:1). Slides were prepared and stained with GIEMSA. G banding was done to analyse chromosomal aberrations [278].

4.2.2.5. Population Doubling Time:

The doubling time of both NIPBC-1 and NIPBC-2 cell lines at passage 35 and 32 respectively, was determined by counting the cell number at regular intervals. On day 0, 1x 10^5 viable cells were seeded in triplicate in each of six well plates (corning, USA) in DMEMF12 culture medium. Cells were counted in triplicate with a Neubauer chamber at exactly 24hr intervals for a series of 10 days after staining with trypan blue dye. The growth curve was plotted and the doubling time was calculated from regression equation of the curve [279-282].

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4.2.2.6. DNA finger printing/STR profiling:

STR profiling of NIPBC-1 and NIPBC-2 cell lines was done using StemElite ID System kit (Promega) as per the manufacturer's instructions. This method allows identification of unique detection of short tandem repeats, seven human STR loci, Amelogenin (for gender identification) and one mouse locus, which include TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, D5S818 and MUS. These loci collectively provide a genetic profile with a random match probability of 1 in 2.92 x 10^9 (while simultaneously providing detection of a 1% fraction of mouse contaminant in a human cell line). The eleven loci are amplified

simultaneously in a single tube and analyzed by capillary electrophoresis on 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

4.2.2.7. Invasion Assay:

To assess the invasive capacity of NIPBC-1 and NIPBC-2 cells, we utilized Corning invasion chambers and coated them with matrigel; MDA-MB-231 cells were used as positive control. Cells were trypsinised and washed twice with PBS before they are transferred to the invasion chamber. Prior to use, chambers were rehydrated with DMEM for 2 hours at 37°C then plated with 5×10^4 cells per well. After 12 hours of incubation, invasion chambers were fixed in 4% paraformaldehyde for 15 minutes, stained with hematoxylin, and washed in PBS. Cancer cells that invaded through the matrigel-coated filter on the lower membrane were manually counted under a microscope. Four randomly chosen fields were counted for each well. The experiment was performed in triplicates.

4.2.2.8. Mycoplasma Detection:

The mycoplasma DNA was detected by the PCR kit Venor GeM (Minerva Biolabs) as per the manufacturer's instructions. This kit is specific for a spectrum of contaminants of cell lines and their biological derivatives belonging to Mycoplasma acholeplasma and ureaplasma species. The primer set is specific to the highly conserved 16S rRNA coding region in the mycoplasma genome. Detection requires as little as 1–5fg of mycoplasma DNA corresponding to 2–5 mycoplasma per sample volume. The resulting PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, visualized under UV light and documented by photography.

4.2.2.9. Mutational Analysis of TP53:

Genomic DNA was isolated from NIPBC-1 and NIPBC-2 cell lines using Gene aid DNA isolation kit as per the manufacturer's instructions. Polymerase chain reaction (PCR) amplified products encompassing exons 2- 3, 4, 5-6, 7, 8, 9, 10 and 11 of the TP53 gene were analyzed for mutations by automated sequencing using 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

4.2.2.10. Study of Stem cell Population

(i) Aldefluor Assay and Flow Cytometry

Aldefluor assay was performed as per the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada) in NIPBC-1, NIPBC-2 and MCF7 breast cancer cell lines. Briefly, single cells obtained from cell cultures were incubated in Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (BAAA, 1μmol/L per 1,000,000 cells), for 30 min at 37 °C. A fraction of cells from each sample, incubated under identical condition in the presence of diethylaminobenzaldehyde (DEAB) was taken as negative control [283]. Flow cytometry was conducted using FACS ARIA II SORP (Special order research product) (Becton Dickinson). ALDEFLUOR florescence was excited at 488nm and florescence emission was detected using a standard FITC 530/30 band pass filter. The sorting gates were established using the negative control [41, 284].

(ii) Identification of $CD44^+/CD24^-$ Breast cancer stem cells by flow cytometry:

Al- Hajj and collegues have demonstrated that expression of stem-cell markers $CD44^+/CD24^{-/low}$ in mammary tumors has prognostic significance [27]. Breast cancer cell line MCF7 was taken as a positive control. To sort tumor cells with CD44 and CD24 markers both NIPBC-1 and NIPBC-2 cell lines were trypsinized to make single cell suspensions. Approximately, $1x10^7$ cells suspended in 1ml of staining buffer were stained with fluorochrome conjugated monoclonal antibodies against human CD44 (PE) and CD24 (FITC) both individually and in combinations. The isotype controls were also added to the cell suspensions as per the manufacturer's instructions and were incubated in the dark for 45 min. Unbound antibody was washed off and cells were analyzed for the presence of $CD44^+/CD24^-$ cell population, no longer than 2 hr post staining on BD FACS ARIA. The sorting gates were established using the isotype matched controls [27].
This study had been approved by institutional ethics committee of Safdarjung Hospital, New Delhi and consent has been obtained from each patient for participating in this study.

4.3. Results:

4.3.1. Establishment of primary cultures:

We have initiated 31 primary cultures using 44 biopsies obtained from patients diagnosed with carcinoma of breast. Out of these primary cultures, 2 cell lines could be successfully purified and propagated for more than 60 passages. Among the two cell lines established, NIPBC-1 was derived from a breast cancer patient aged 39 years and NIPBC-2 from a 38 year old patient. The breast tumor in both the cases has been diagnosed as Infiltrating breast cancer NOS type, grade IIb and grade III respectively. The two cell lines were established by enzymatic disaggregation followed by differential trypsinization. NIPBC-1 was initiated after enzymatic disaggregation of tumor tissue with trypsin. Cells were seeded onto the surface of a flask and cells which adhered to the surface by day 1, reached confluency by day 10; the cells were then subjected to differential trypsinization, until a pure epithelial cell population is remained in the flask. NIPBC-1 cells are spindle shaped cells which grow sparse, do not grow in layers and adhere strongly to the surface (Figure 4.2). The second cell line NIPBC-2 was initiated by enzymatic disaggregation with both trypsin and collagenase; the cells adhered to the surface after day 3, and formed cell aggregates consisting of epithelioid cells. These cells proliferated and occupied the whole surface by day 8. They formed distinct large epithelial colonies surrounded by fibroblasts, which were further enriched by selective scraping of the fibroblasts. NIPBC-2 cells are cuboidal cells which form multilayers. They are fast growing, and easily get detached upon trypsinization. NIPBC-1 and NIPBC-2 cell lines were so far passaged for 85 and 66 times respectively in our laboratory (Figure 4.3).

4.3.2. Expression of Epithelial and biological markers:

Expression of epithelial markers EMA, Cytokeratin-18, 5/6, mesenchymal marker vimentin, Estrogen receptor and P53 was studied in the established cultures by immunoflorescence (Figure 4.4 and 4.5) and immunocytochemistry (data not shown) to determine their histogenesis. Both NIPBC-1 and NIPBC-2 cells showed immunonegativity for cytokeratin 5/6 and are found to be non-basal. Both the cell lines were found to be triple negative (ER -ve, PR -ve and HER2/neu –ve). While

the NIPBC-1 cell line showed over expression of cytoplasmic Muc1, but was found p53 negative. NIPBC-2 cell line did not show expression of Muc1 but showed a strong nuclear positivity for p53. Further we have analyzed expression of cell cycle markers viz., Ki67, p53 and p21 proteins by flow cytometry. Ki67 expression was observed in both NIPBC-1 and NIPBC-2 (Figure 4.6), whereas p53 expression was found only in NIPBC-2 confirming the immunofluorescence finding. Both the cell lines demonstrated only negligible amounts of p21 (Figure 4.7 and 4.8).

4.3.3. Electron microscopic analysis:

The epithelial lineage of both NIPBC-1 and NIPBC-2 cells was further confirmed by ultrastructure study of the cells by transmission electron microscopy. The cells from both the cell lines showed presence of hyperchromatic vesicular nucleus with 2 or 3 nucleoli. The cytoplasm showed presence of ribosomes, bundles of microfilaments and secretary vesicles. The cells were polygonal and attached to each other with tight junctions and desmosomes. Few microvilli were also present at the cell surface (Figure 4.9).

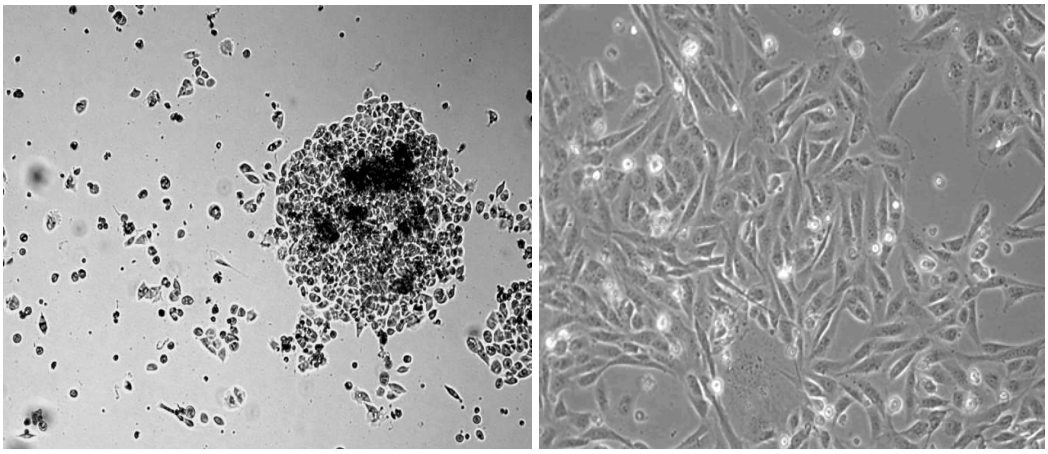
4.3.4. Anchorage Independent growth:

NIPBC-1 and NIPBC-2 when plated as single suspension on 0.3% agar, formed large colonies (40-100) after 2 weeks, MCF7 cell lines were used as positive control for this experiment which also formed large colonies on agar (Figure 4.10).

4.3.5. Population Doubling Time:

Population doubling time of cell lines NIPBC-1 and NIPBC-2 cell lines were determined as described in materials and methods. The doubling time of NIPBC-1 cell line was found to be 33.25hrs, while that of NIPBC-2 was 31.56hrs (Figure 4.11).

(a) (b)



(c)

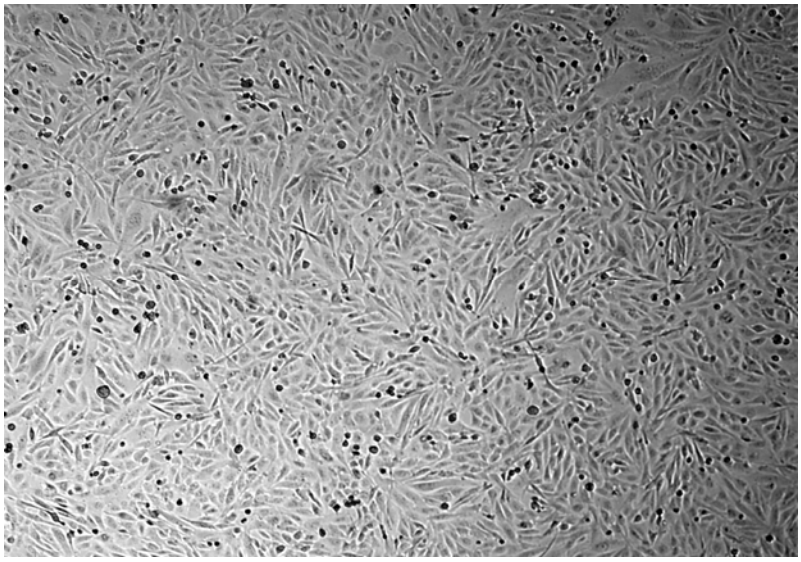
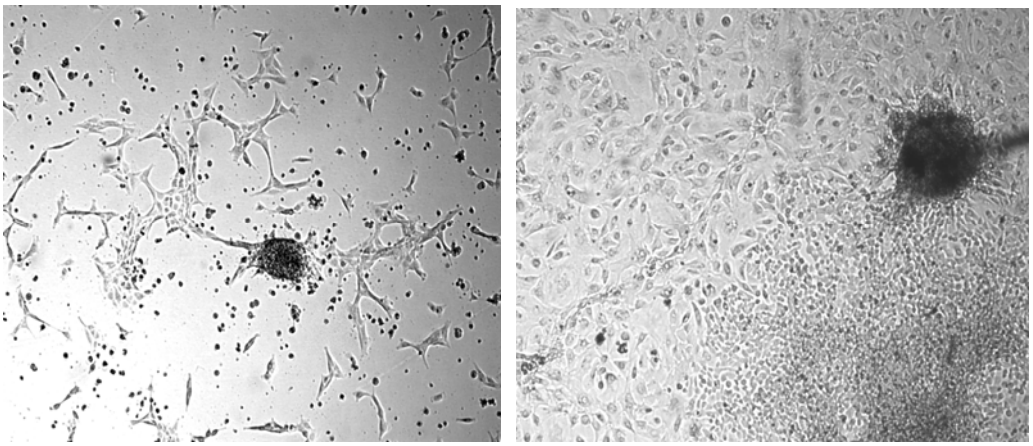


Figure 4.2. a, b. illustrates the establishment of breast cancer cell line NIPBC-1 at different passages. (a) by day 2 different cell types started adhering to the substratum of the flask. (Original magnification x 40) (b) primary culture at passage 5 showing a mixed population of epithelial cells and fibroblasts (Original magnification x 40) (c) at passage 12 removal of fibroblasts by differential trypsinisation yielded a pure population of epithelial cells (Original magnification x 40)

(a) (b)



(c)

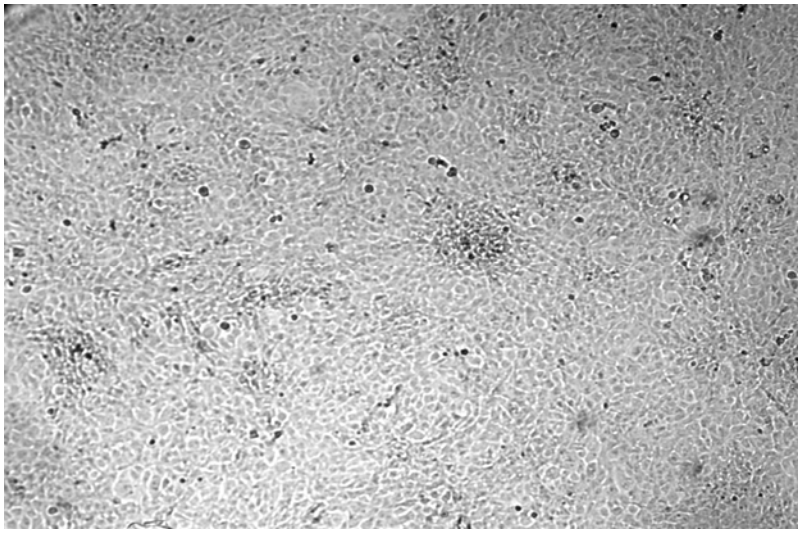


Figure 4.3. a, b illustrates the establishment of breast cancer cell line NIPBC-2 at various passages. (a) Cells from the enzyme digested tissue adhered to the surface after day 3, and formed cell aggregates consisting of epithelioid cells (Original magnification x 40). (b) distinct large epithelial colonies surrounded by fibroblasts formed by 10 days. The fibroblasts were scraped manually (Original magnification x 40). (c) at passage 14 removal of fibroblasts by manual scraping and differential trypsinisation yielded a pure population of epithelial cells (Original magnification x 200).

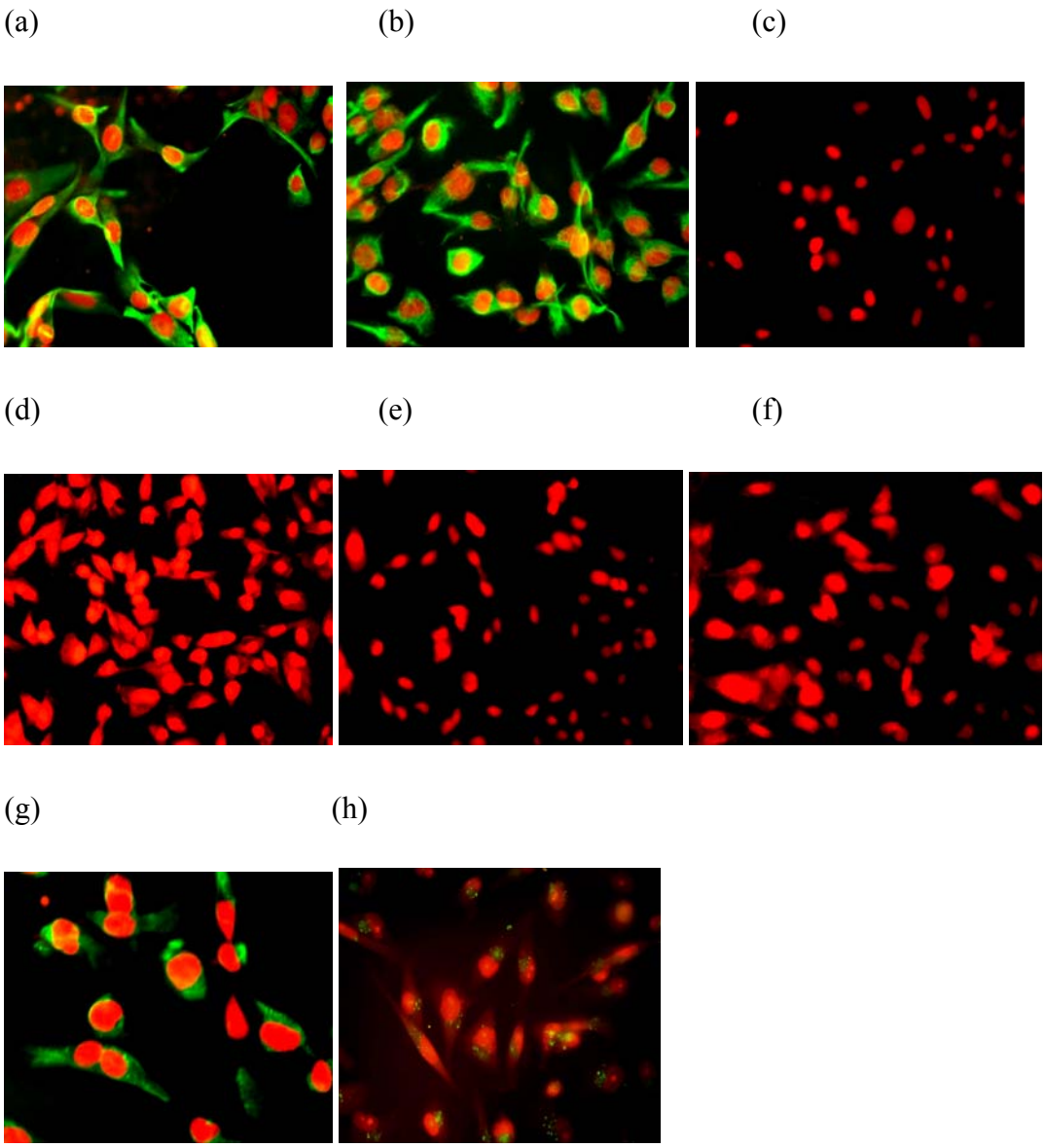
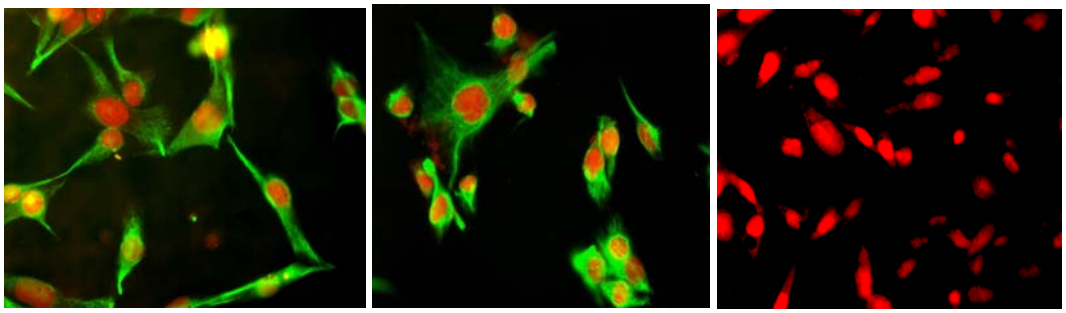
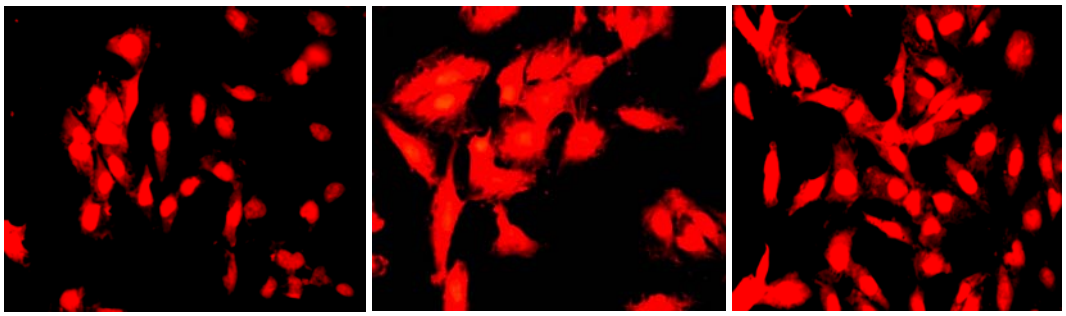


Figure 4.4. Expression of biological markers in the established breast cancer cell line NIPBC-1. (a) Epithelial membrane antigen, (b) Cytokeratin 18, (c) Cytokeratin 5/6, (d) Estrogen receptor, (e) Progesterone receptor, (f) HER2/neu (g) P53 and (h) Vimentin.

(a) (b) (c)



(d) (e) (f)



(g)

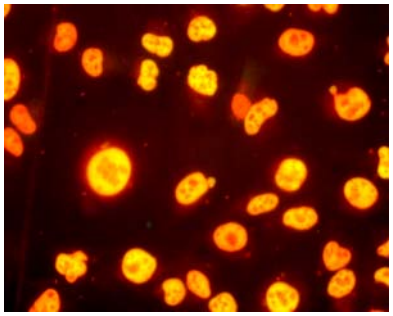


Figure 4.5. Expression of biological markers in the established breast cancer cell line NIPBC-2. (a) Epithelial membrane antigen, (b) Cytokeratin 18, (c) Cytokeratin 5/6, (d) Estrogen receptor, (e) Progesterone receptor, (f) HER2/neu and (g) P53

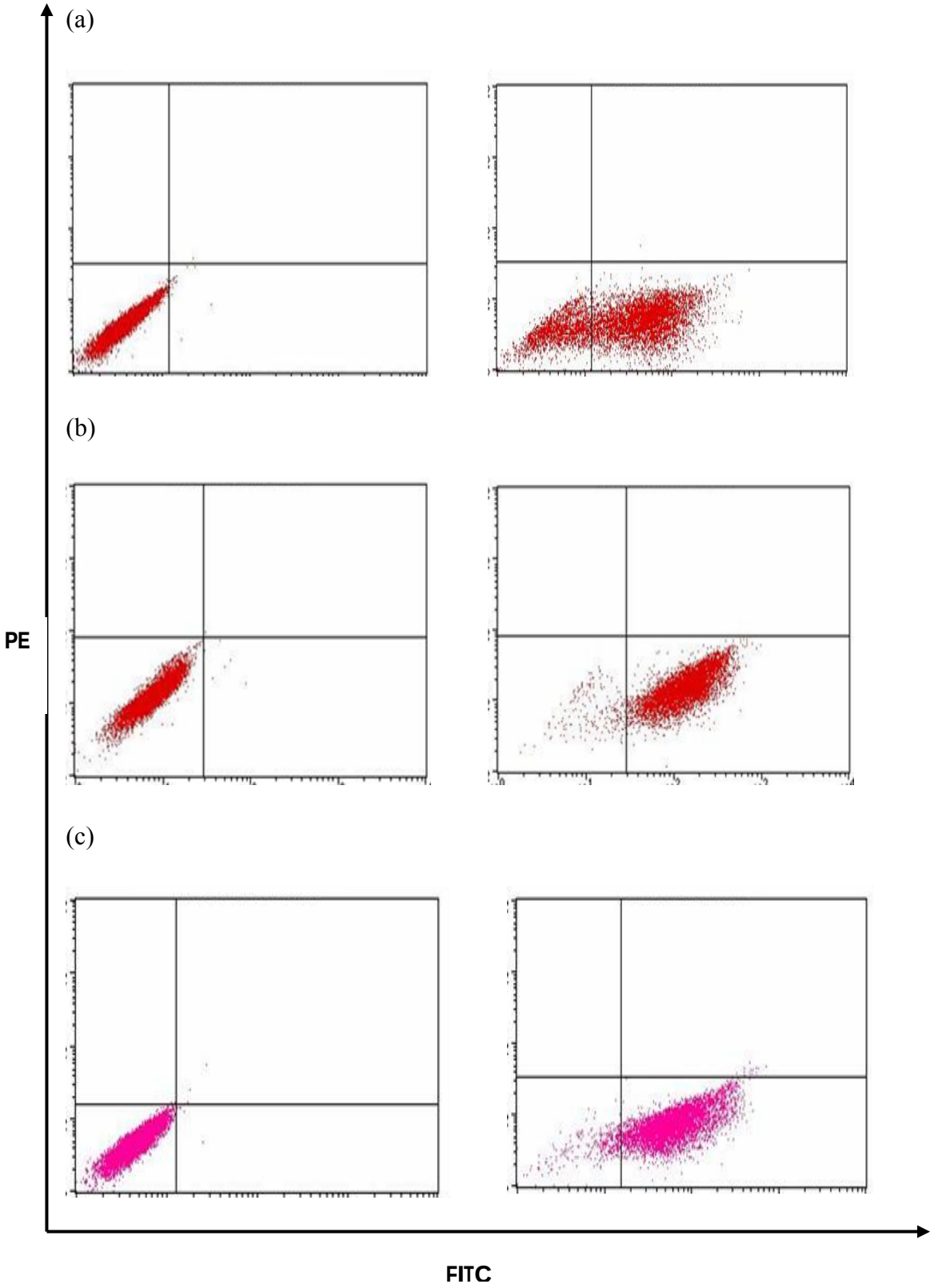


Figure 4.6. Expression of cell cycle marker Ki67 by FACS in MCF7, NIPBC-1 and NIPBC-2 cell lines along with their isotype controls. (a) MCF7 (b) NIPBC-1 (73.01%) (c) NIPBC-2 (94.11%).

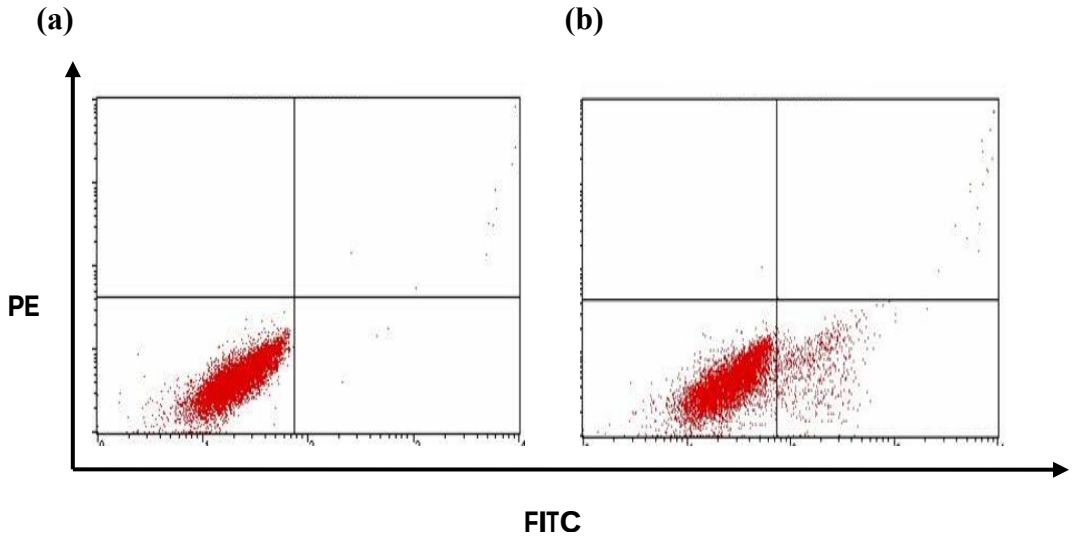


Figure 4.7. Expression of cell cycle markers by FACS in NIPBC-1 Cell line. (a) P53 (0.26%) and (b) P21 (11.13%)

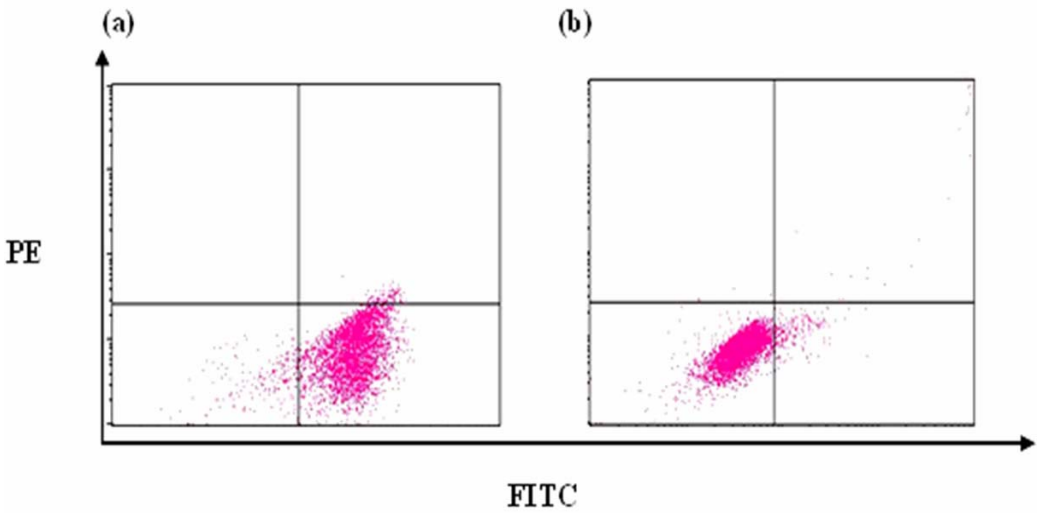


Figure 4.8. Expression of cell cycle markers by FACS in NIPBC-2 Cell line. (a) P53 (91.80%) (b) P21 (4.25%)

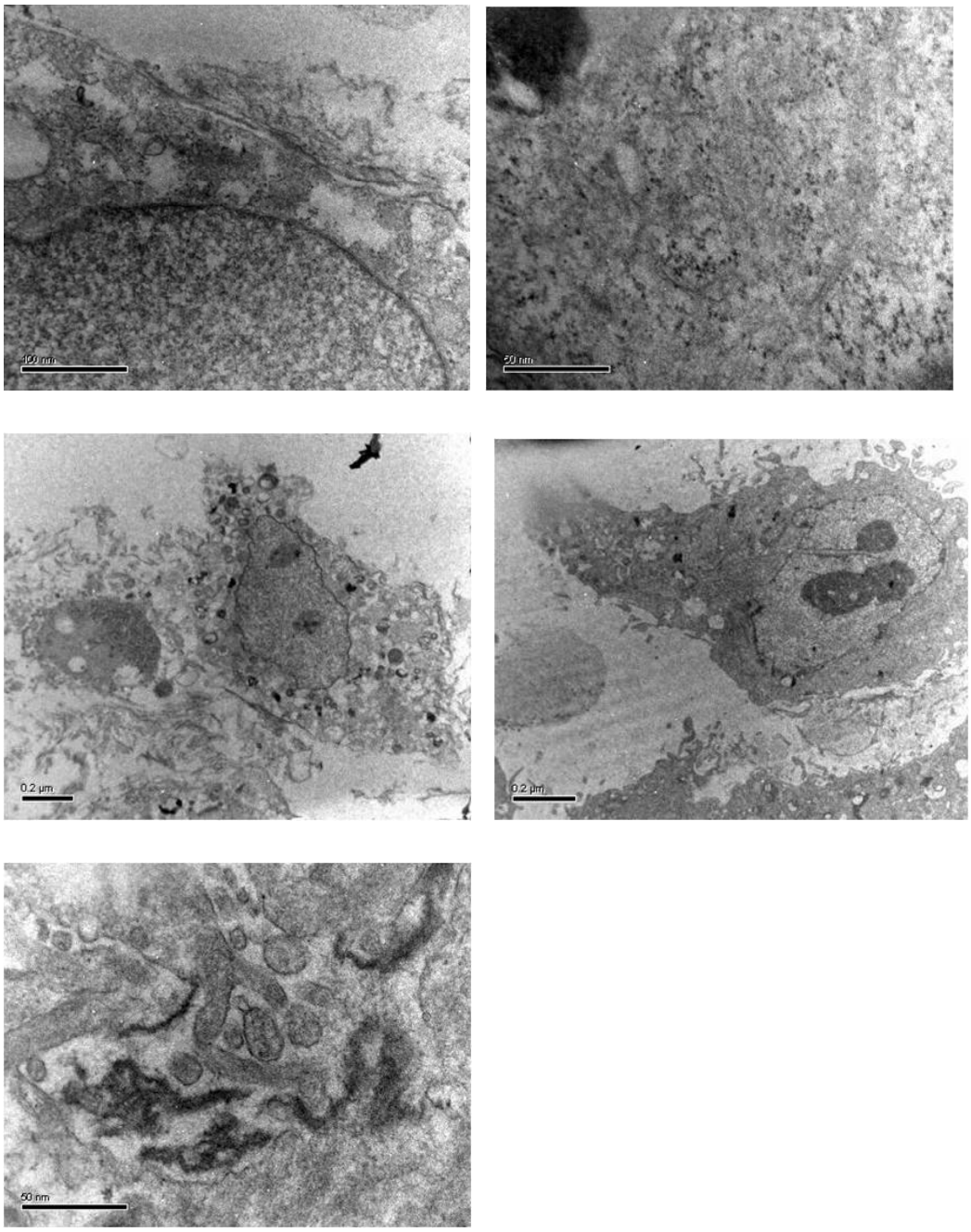
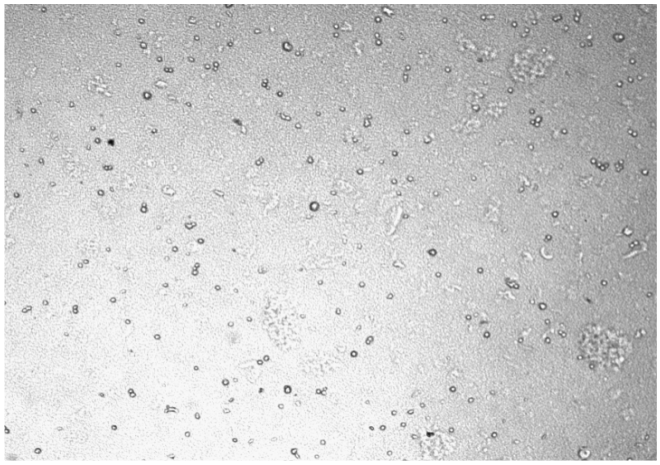
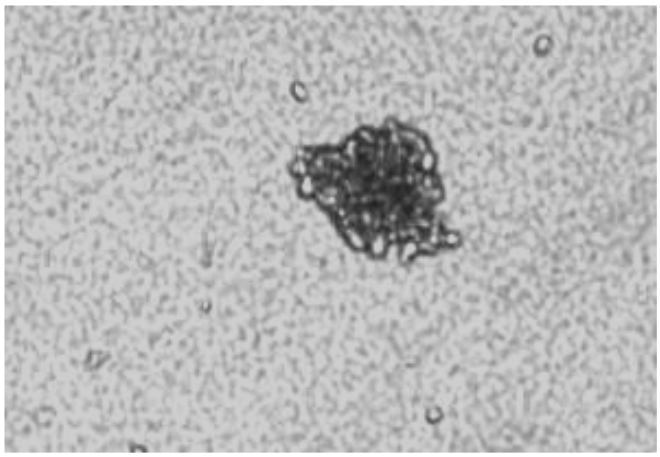


Figure 4.9. Electron micrographs of the established cell lines

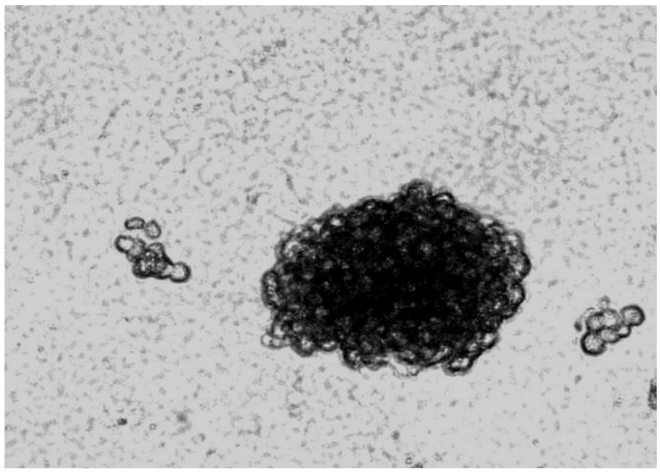
(a) (i)



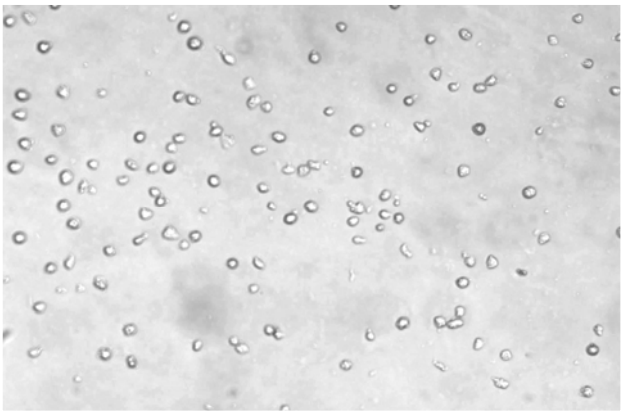
(ii)



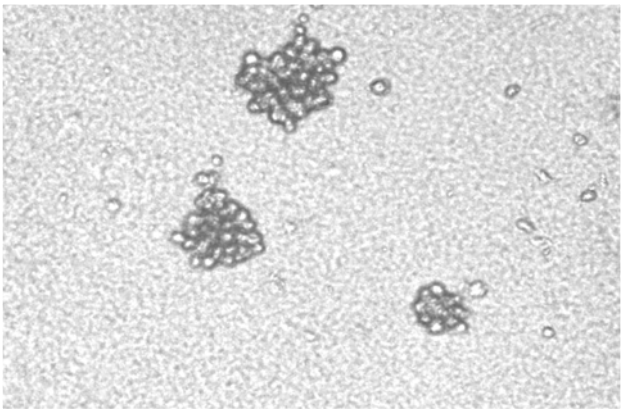
(iii)



(b) (i)



(ii)



(iii)

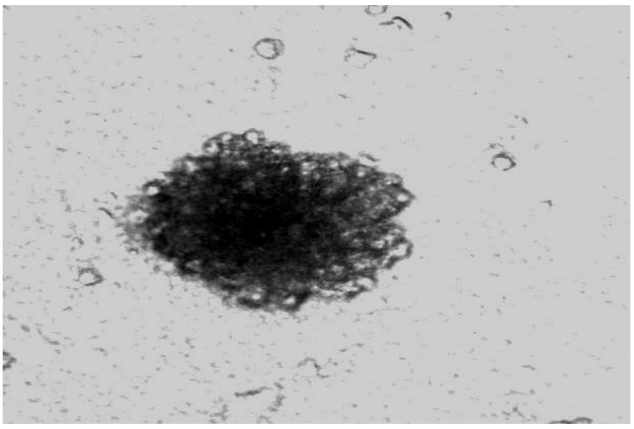
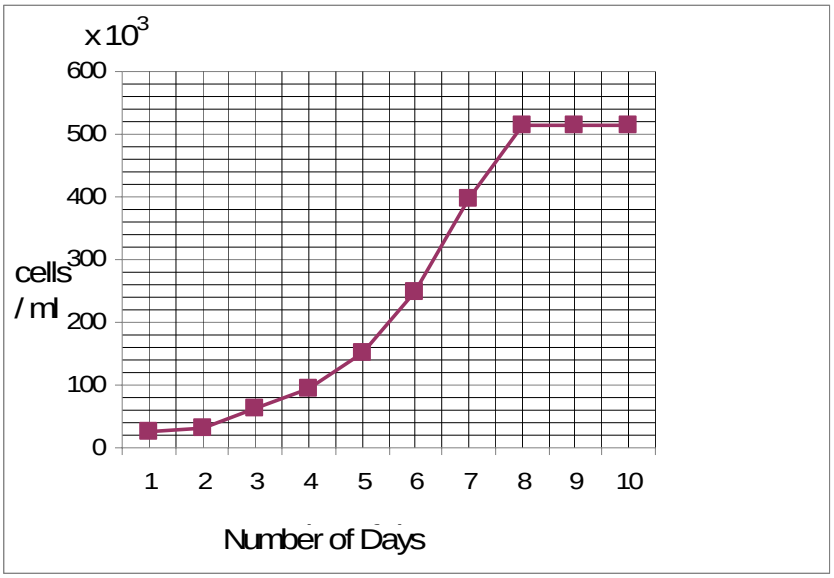


Figure 4.10. Representative pictures of colonies formed in anchorage independent growth by (a) NIPBC-1 and (b) NIPBC-2 cell lines at (i) day 1, (ii) day 7 and (iii) day 14

(a)



(b)

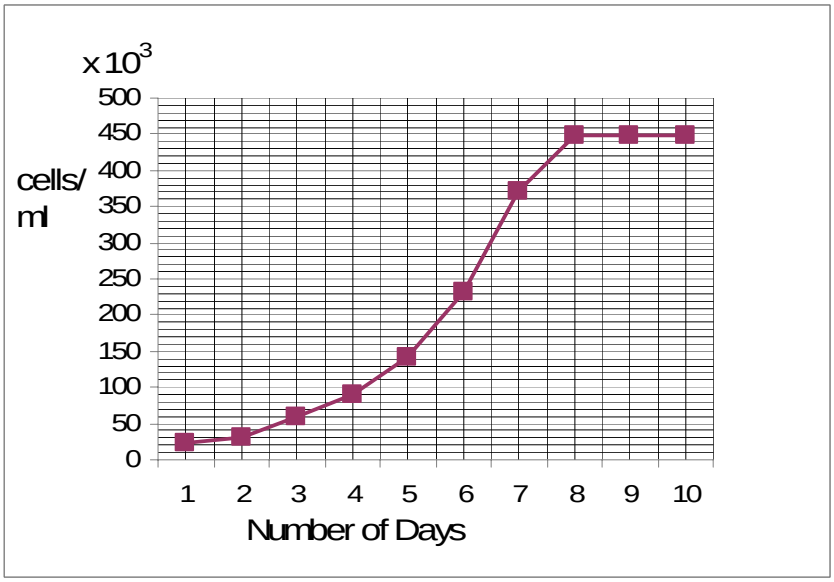


Figure 4.11. Population doubling time: Cells were plated in 6-well plates at a plating density of 1 x 105cm2 in DMEM growth medium, supplemented with 10% FBS. Growth medium was renewed every 3 days. Cell counts were performed on the days indicated. Each point represents the mean of three different determinations made in triplicate. The population doubling times of the established cell lines (a) NIPBC-1 and (b) NIPBC-2 were calculated to be 33.25hrs and 31.56hrs respectively.

4.3.6. Karyotype analysis

Karyotype analysis of NIPBC-1 and NIPBC-2 cells has shown that both the cell lines possess aneuploidy. Chromosomes 7, 9, X and 11 showed deletions in various regions in both the cell lines. Cytogenetic analysis has shown multiple rearrangements. NIPBC-1 was near tetraploid with a modal number of 58 to 62 chromosomes, most of the chromosomes exhibited several translocations and marker chromosomes; rearrangements like t(14:15) (q12:q12) and i(17q) were found commonly in these cells; Isochromosomes 17q was the most common aberration identified in NIPBC-1. NIPBC-2 cell line was also found to be aneuploid with nearly tetraploid to pentaploid complement and the chromosomal numbers ranged from 107 to110 (Figure 4.12. and 4.13.). No significant karyotype changes were found in the karyotypic analysis among the early and late passages of both the cell lines; indicating that both NIPBC-1 and NIPBC-2 are stable cell lines.

4.3.8. Invasion Assay:

To examine the invasion capacity of the two cell lines established in the present study, we have carried out invasion assay on matrigel coated membrane inserts. We found invaded cells on the other side of the membrane upon overnight incubation with chemo attractant (FBS). Breast cancer cell line MDA-MB231 was taken as positive control. The number of cells invading the matrigel matrix are counted in atleast 4 randomly choosen fields per well. The numbers of MDA-MB231, NIPBC-1 and NIPBC-2 cells that invaded through the basement membrane were 527 ± 45.9020, 409.3 ± 32.3161, 290.6 ± 44.2417 respectively (Figure 4.14. a-c). The number of NIPBC-1 cells that invaded through the basement membrane was significantly higher than that of NIPBC-2 cell line ($p= 0.0407$) (Figure 4.14. d).

4.3.9. Test for Mycoplasma contamination:

Various passages of both NIPBC-1 and NIPBC-2 cell lines were tested for the presence of mycoplasma by a PCR based method using “Venor GeM Mycoplasma detection kit”. Both the cell lines have been found to be free of mycoplasma contamination. (Figure 4.15)

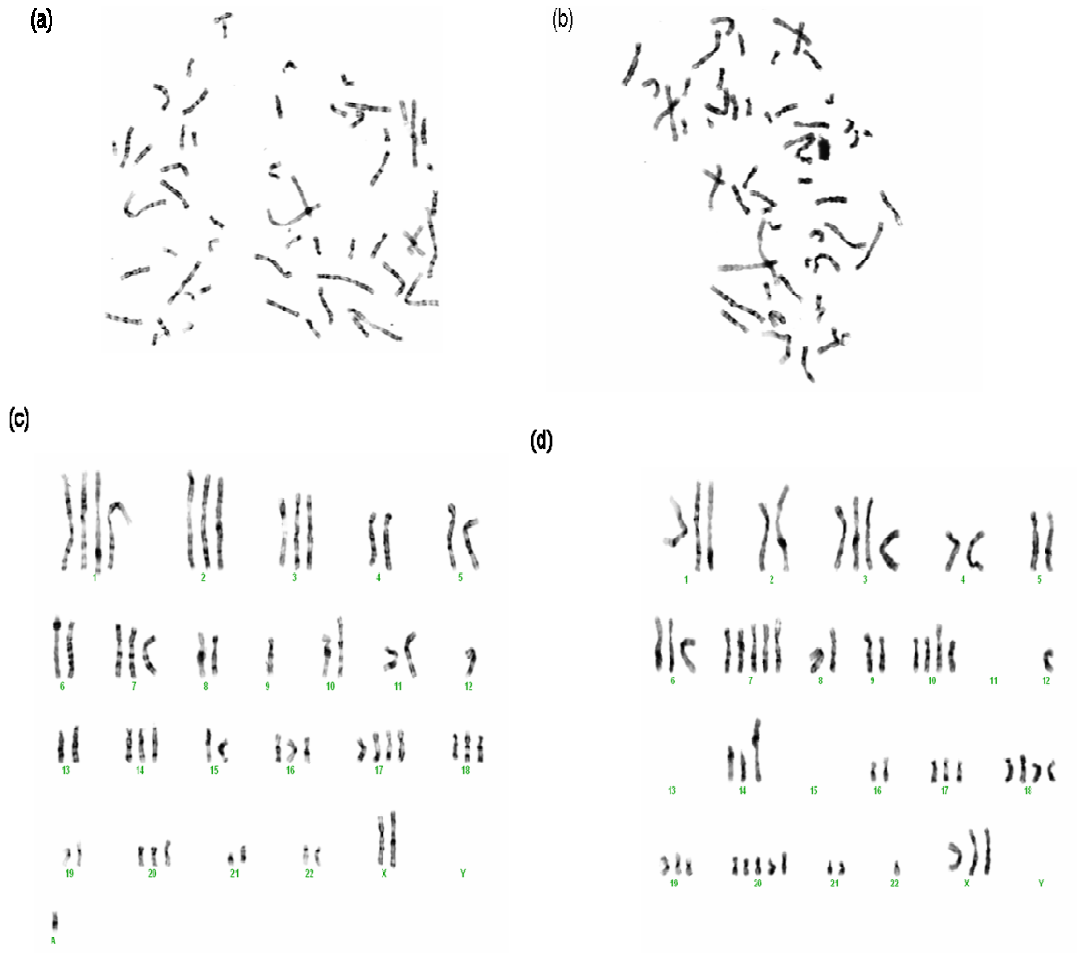
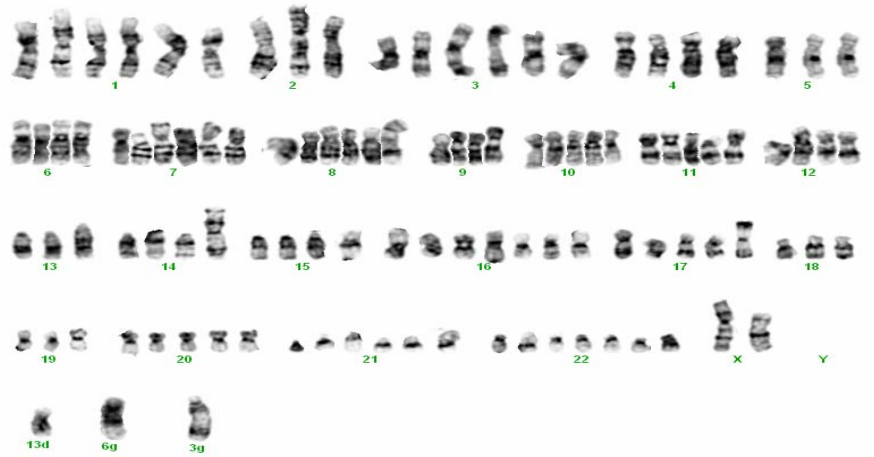


Figure 4.12. Representative metaphases (a, b) of NIPBC-1 cells, at passages 20 and 65, with trypsin-giemsa banding. Karyotypes (c, d) of the above metaphases showing near tetraploidy with a modal number of 58 to 62 chromosomes.

(a) (b)

(c)



(d)

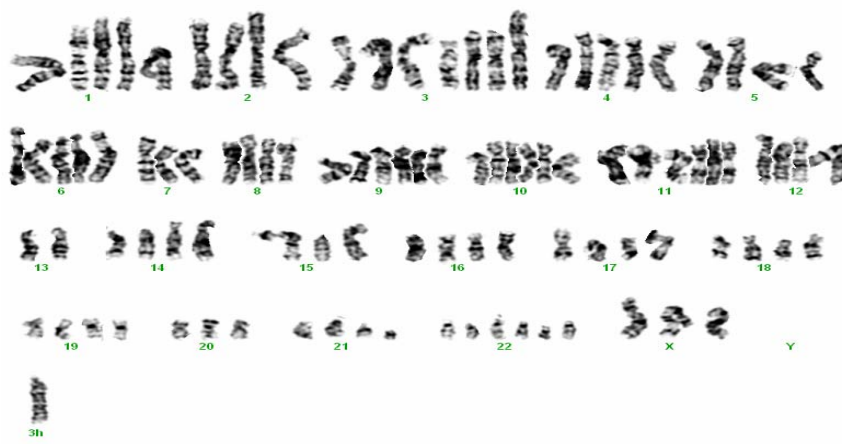


Figure 4.13. Representative metaphases (a, b) of NIPBC-2 cells, at passages 15 and 52, with trypsin-giemsa banding. Karyotypes (c, d) of the above metaphases showing near tetraploidy with a modal number of 58 to 62 chromosomes.

4.3.7. DNA finger print/STR Analysis:

STR profiling of standard STR markers [285] was done for both NIPBC-1 and NIPBC-2 cell lines established distinct profiles of both the cell lines. (Table 4.2) Both the cell lines did not show any peak for the mouse marker MUS. The marker Amelogenin has shown only a single peak with an allele size of 104, which shows that both the cell lines are from female origin with XX chromosomes. Further, this analysis established the fact that there is no cross contamination among the two cell lines.

Marker	**NIPBC-1**	**NIPBC-2**
D5S818	11	11
Mouse	-	-
D13S317	12	12
D7S820	10	8, 12
D16S539	11	9, 12
CSF1PO	10, 11	10
AMEL	X, X	X, X
VWA	12	12
TPOX	8, 13	8

Table 4.2. STR profiling of NIPBC-1 and NIPBC-2 cell lines

4.3.10. TP53 Mutational Analysis:

No mutation was found in the coding regions of both NIPBC-1 and NIPBC-2 cell lines. NIPBC-2 cell line has heterozygous C/G, g.417 C>G (NM_000546.5), at codon72of exon 4, resulting in p.P72R (Pro/Arg allele); While, NIPBC-1 has homozygous Pro/Pro allele (no change), at codon 72 (Figure 4.16) (Table 4.3).

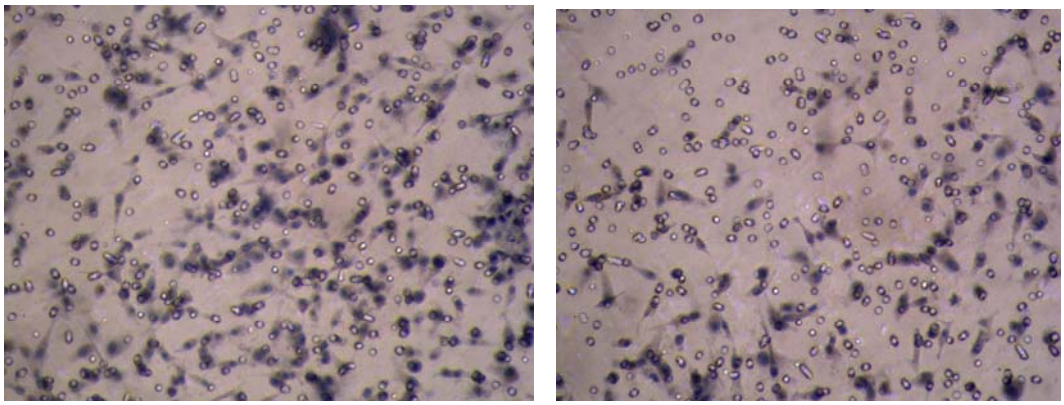
4.3.11. Expression of Breast cancer stem cells by flow cytometry:

To obtain breast CSCs, we have stained and sorted both NIPBC-1 and NIPBC-2 cell lines using antibodies against CD44 and CD24 cell surface markers taking MCF7 breast cancer cell line as positive control. We could detect 0.2% and 0.1% of $CD44^{+}/CD24^{-}$ breast cancer stem cells in NIPBC-1 and NIPBC-2 cell lines respectively (Figure 4.17).

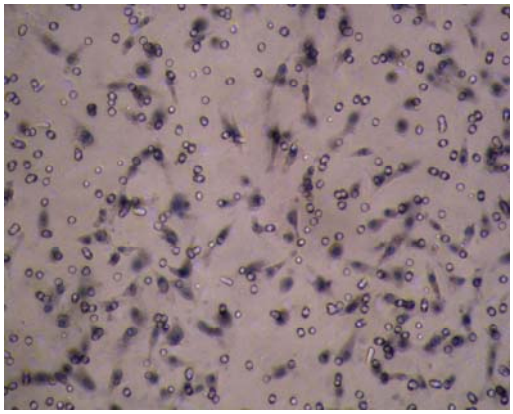
4.4. Discussion:

We have established two triple negative breast cancer cell lines NIPBC-1 and NIPBC-2 from primary tumors of two young breast cancer patients (39 and 38 yrs old) both showing nonbasal origin. In India premenopausal patients constitute about 50% of all patients (Agarwal *et a.l*, 2007). Early-onset breast cancer may, in part, be biologically different from breast cancer patients in older patients [286]. Family history contributes to only 20% of the early onset cases whereas factors responsible for the rest of the breast cancer cases in young women are not known [287]. Difference in clinical behavior and molecular profile of early onset breast cancer suggest the need for understanding the risk factors and molecular mechanisms involved in development of breast cancer in young women. There are few breast cancer cell lines available (<20%) from patients <40 years of age. The two cell lines established in the present study NIPBC-1 and NIPBC-2 were derived from breast cancer patients with the age 39 years and 38 years respectively, and represent breast cancers that occur at early age; hence may serve as *in vitro* models to study the early onset breast cancers in Indian women.

(a) (b)



(c)



(d)

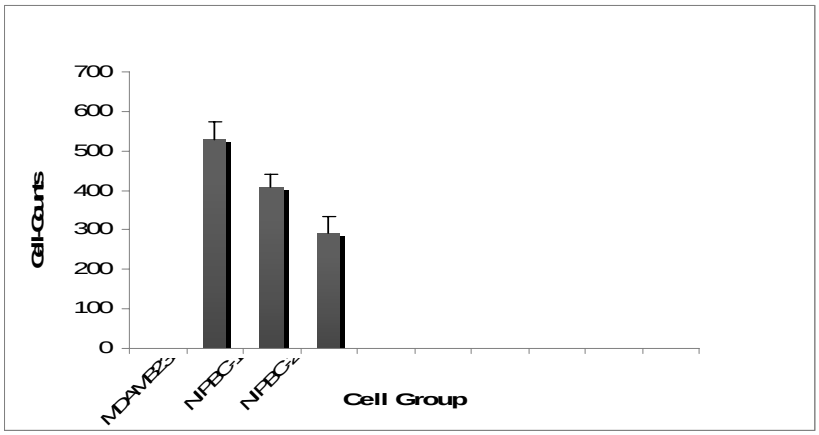


Figure 4.14. Invasion assay of NIPBC-1 and NIPBC-2 cell lines. Representative pictures of (a) MDA MB 231 (positive control), (b) NIPBC-1 and (c) NIPBC-2 cells invaded through Matrigel. (d) Cell number quantification of invasion.

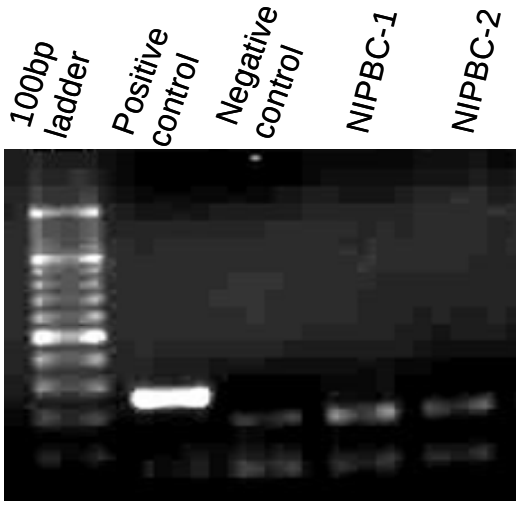


Figure 4.15. Test for mycoplasma contamination in NIPBC-1 and NIPBC-2 cell lines

The success rate of establishing cell lines in present study is 4.5% ie., 2 cell lines using 44 primary tumors which is comparable to other studies in breast cancer where also low success rate had been reported [124]. The epithelial origin of both the cell lines, NIPBC-1 and NIPBC-2 has been confirmed by electron microscopic examination and immunofluorescence. Both NIPBC-1 and NIPBC-2 cells are negative for cytokeratin 5/6 and positive for EMA, demonstrating their non-basal epithelial nature. NIPBC-1 cells showed over expression of MUC1 cells, suggesting their transformed nature [288, 289], further it has shown punctate vimentin positivity suggesting metaplastic behavior of these cells, which is corroborating with their spindle shape. Vimentin has been previously linked to the metaplastic potential of cancer cells as its increased expression has been demonstrated to be a marker of epithelial mesenchymal transition (EMT). Also Willipinski-Stapelfeldt *et al*., [290] stained more than 2500 primary breast tumors and demonstrated that approximately 35% of hormone receptor negative tumors expressed vimentin but only 7% of hormone receptor positive tumors expressed vimentin. Also Connie L. Sommers *et al*., [291] reported that vimentin was expressed by more than one-half of the hormone-independent breast carcinoma cell lines tested but not by the hormone-dependent cell lines. Moreover it is a well

established fact that most eukaryotic cells start expressing vimentin when brought into tissue culture [292, 293] .

Vimentin which was originally identified as an intermediate filament protein present only as an intracellular component has been detected recently on the surface of cancer cells but not on healthy cells in a punctate distribution pattern. Nicole F. Steinmetz *et al*., [294, 295] have demonstrated that surface vimentin can be used as a common marker to detect highly metastatic cancer cells. They have also examined the coexpression of surface vimentin with the CD44 and CD133 stem- or progenitor cell marker proteins and demonstrated that Cowpea mosaic virus (CPMV) nanoparticles can bind to the surface domains of vimentin thereby facilitating drug targeting in prostate cancer. NIPBC-1 cell line which expresses punctate surface vimentin can be used as a model for creating nanoparticle- or antibody- cancer therapeutic agents capable of targeting vimentin in combination with other surface markers to prevent cancer metastasis as well as kill cancer stem cells. Also it can be used as a model to detect highly metastatic cancer cells.

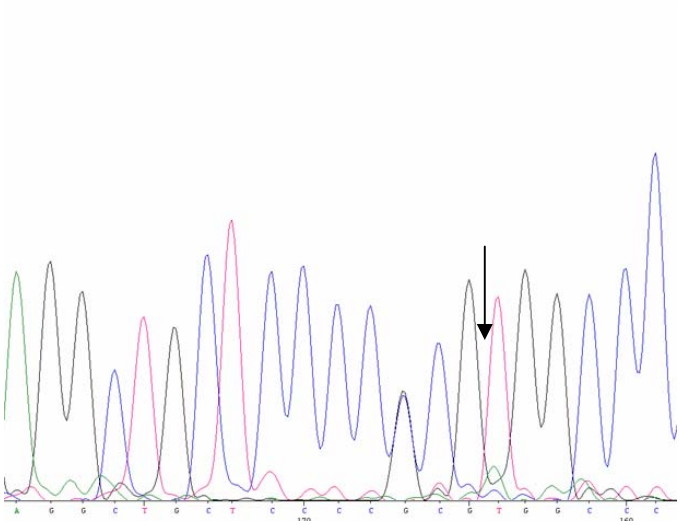


Figure 4.16: TP53 mutational analysis. NIPBC-2 cell line has heterozygous C/G, g.417 C>G (NM_000546.5), at codon72 of exon 4, resulting in p.P72R (Pro/Arg allele).

Name of the Exon	Primer sequence	Product size
Exon 2-3	tctcatgctggatccccactagtcagaggaccaggtcctc	344 bp
Exon 4	tgctcttttcacccatctacatacggccaggcattgaagt	353 bp
Exon 5-6	tgttcacttgtgccctgactttaacccctcctcccagaga	467 bp
Exon 7	cttgccacaggtctccccaa aggggtcagaggcaagcaga	196 bp
Exon 8	gacaagggtggttgggagta taactgcacccttggtctcc	287 bp
Exon 8-9	ttgggagtagatggagcctagtgttagactggaaacttt	445 bp
Exon 10	caattgtaacttgaaccatcggatgagaatggaatcctat	260 bp
Exon 11	agaccctctcactcatgtgatgacgcacacctattgcaag	245 bp

Table 4.3 List of primers used for p53 sequencing

The ultra structural features confirming epithelial origin of both the cell lines include presence of bundles of microfilaments, secretary vesicles, tight junctions, desmosomes, and granular cytoplasm with numerous ribosomes. Both NIPBC-1 and NIPBC-2 cell lines are negative for estrogen receptor (ER), progesterone receptor (PR) and HER2/neu, and hence represent triple negative breast cancers (TNBC), which are considered to be aggressive tumors having higher histological grade, worse prognosis and occur mostly in younger women [296-298]. Most of the triple negative tumors are of basal type [298], however, the two cell lines we have established are triple negative but are of non-basal origin which makes them unique. It has been observed that Indian breast cancer patients have a higher tendency to have triple negative tumors as compared to western patients. Incidence of triple negative breast cancer in Indian breast cancer patients has been reported

between 19.9- to 24.8% compared to 15% in the SEER, and there is significant correlation of TN Breast cancer with younger age (<35 years, P≤ 0.003) and high level of p53 mutations (P<0.001)). The younger the age of the patient, the greater are the chances of the cancer being ER negative, with 63.5% of patients under 50 years of age having ER negative tumors, and 33% of patients under 50 years of age having TNBC [299]. Khokhar *et al*., [300] reported that Estrogen (ER) and progesterone receptors (PR) are found positive in only 20-45% of Indian patients. ER-positive rates were reported to be lower in Indian patients than those in western countries. Rao *et al*., [301] reported that 33% of triple negative phenotype breast carcinomas show expression of basal markers (CK5/6 and/or over-expression of EGFR) and that "Triple negative" status cannot be used as a surrogate for "basal marker expression".

NIPBC-1 and NIPBC-2 both formed large colonies on soft agar confirming the transformed nature of both the two established cell lines since the ability to form anchorage independent colonies is related to transformed nature of the cells [302]. Further, these cell lines showed positivity for invasion on matrigel confirming their malignant nature.

Both NIPBC-1 and NIPBC-2 cell lines have shown aneuploidy upon karyotyping, which is a feature of neoplastic cells, supporting their neoplastic origin. Further they exhibited several translocations and rearrangements pointing towards their neoplastic nature. The karyotypes of both NIPBC-1 and NIPBC-2 cell lines exhibit isochromosome 17q. The isochromosome 17, i(17q) is a relatively common karyotype abnormality, that results in loss of the short arm (17p) and duplication of the long arm (17q) leading to a single copy of 17p and three copies of 17q, that has been observed in solid tumors such as medulloblastoma, gastric cancer, bladder and breast cancers, associated with tumor development and progression [303] [304]. This is linked to poor survival outcome due to the complex conditions of two important prognostic determinants: loss of tumor suppressors (chromosome 17p) and high expression of oncogenes c-myc (MYCC) or N-myc (MYCN) [305]. Chromosomal gains/loses among the early and late passages were observed. The karyotypes of the late passages of cell lines showed less variability of numerical

aberrations apparently due to clonal adaptation to in vitro conditions. However we would like to point out that cell lines are autonomous dynamic systems with an unlimited lifespan in culture. Numerous studies [306] have reported changes of cell line Karyotype during prolonged cultivation due to changes of culture conditions. It was also demonstrated that even in characterized cell lines, because these cells are continually evolving, there is heterogeneity in karyotypes within the same culture [307, 308].

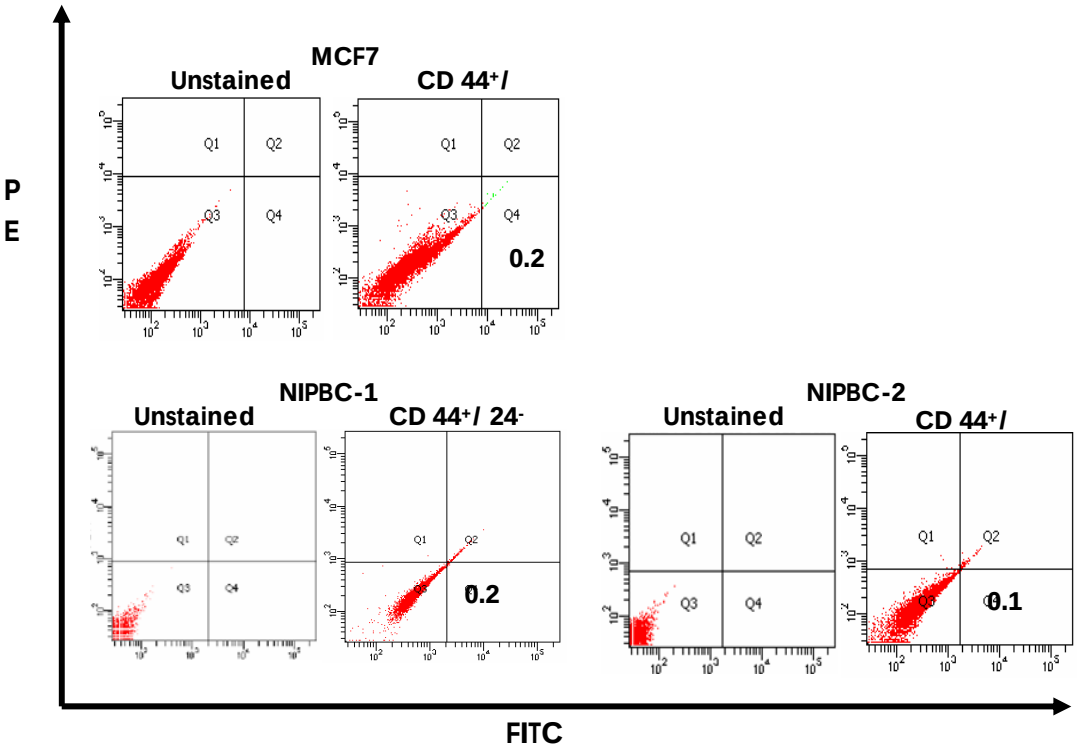


Figure 4.17. Flow cytometry sorting of MCF7, NIPBC-1 and NIPBC-2 cells using CD44 and CD24 markers. Cells were analyzed by fluorescence-activated cell sorting (FACS) using anti-CD44 and anti-CD24 antibodies.

Mutations in tumor suppressor gene P53 genes are significant contributing factors for breast carcinogenesis; hence we have screened these cell lines for mutations/ polymorphisms in these genes. Genetic mutation or allelic polymorphisms in p53 gene are the most common genetic changes associated with much cancer susceptibility in human cancers, such as cervical cancer, breast cancer, lung and colorectal cancer [309]. Although no mutation in coding regions of p53 gene has been noticed in both NIPBC-1 and NIPBC-2 cell lines, however a C/G

polymorphism, g.417 C>G, at codon 72 has been found in NIPBC-2 cell line leading to arginine (A72) being replaced by proline (P72). Numerous polymorphisms in the wild-type p53 have been reported in both the coding and noncoding regions [310]. Of the five polymorphisms described in the coding region, polymorphisms in codon 47 and 72 in exon 4 are functionally well characterized. More common of the two, codon 72 polymorphism is a single-base substitution of cytosine for guanine, leading to arginine (A72) being replaced by proline (P72) [310], and this has been reported to be associated with the risk of several cancers [311-315].The distribution of the three genotypes (Arg/Arg, Arg/Pro and Pro/Pro) depends largely on the ethnic composition of the studied population [316]. Even though, the *p53Arg72* and *p53Pro72* proteins do not differ in their ability to bind to DNA, they bind to components of different transcriptional machinery, influencing differential susceptibility to cancer [317, 318]. The p53Arg72 protein induces apoptosis faster and suppresses transformation more efficiently than the p53Pro72 protein [309, 319]. The p53 Arg72Arg polymorphism may be used as a stratification marker in screening individuals at a high risk of breast cancer [320, 321].

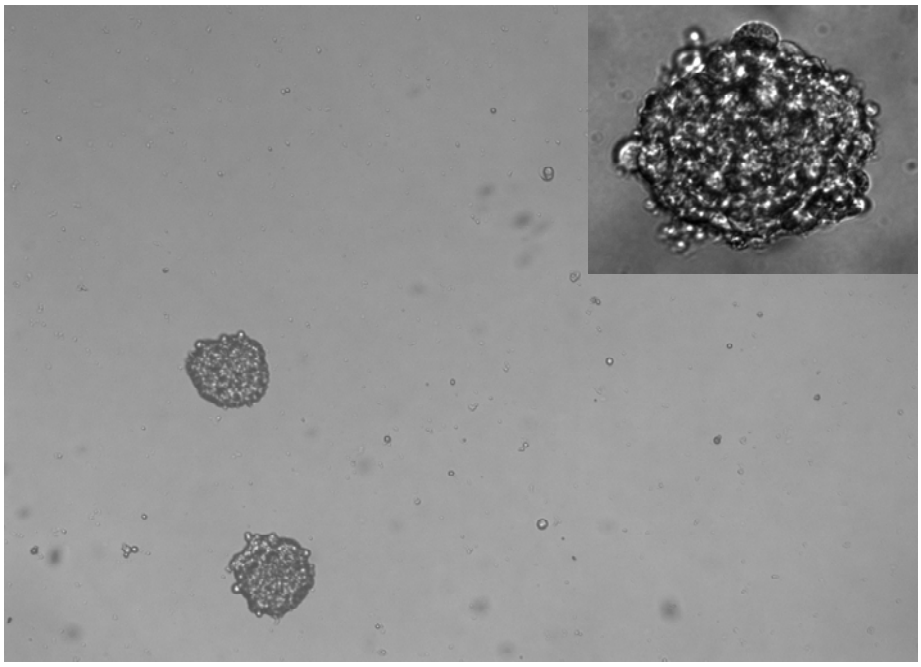


Figure 4.18. Representative pictures of mammospheres formed by NIPBC1 & NIPBC-2 cell lines. (Magnification- 40x, inset mag- 100x)

CSCs have been isolated from many solid tumors, including breast [26, 27], pancreatic [28, 29], brain [30, 31], colon [32-34], liver [35], head/neck [36], ovarian [37, 38], and melanoma [39, 40]. CSCs were first isolated and characterized by Al-Hajj *et al.* using the cell surface marker $CD44^{+}/CD24^{-/low}/Lin-$ and recapitulated the heterogeneity of the original tumor by injecting them into nude mice [27]. Both NIPBC-1 and NIPBC-2 cell lines showed the presence of 0.2% and 0.1% of $CD44^{+}/CD24^{-}$ breast cancer stem cells respectively which is relatively similar to the commercially available breast cancer cell line. When plated in serum free, non-adherent conditions as described earlier [283], both NIPBC-1 and NIPBC-2 cell lines formed mammospheres (Figure. 4.18) and could be further passaged for three generations.

Molecular analysis of the cell lines was done to identify unique properties of the cell lines. Short tandem repeat (STR) profiling was done to ensure the integrity of human cell lines. The STR profile of NIPBC-1 and 2 found to be unique and do not match among themselves and also with other cell lines in the laboratory that are being used, which shows these two cell lines are novel. Although a number of normal and breast cancer cell lines established from western patients are available, there is a paucity of cell lines established from Indian breast cancer patients. So far only two breast cancer cell lines have been established from late onset breast cancer patients (>60 years) using primary tumors [275], hence the two cell lines we have established in the present study are unique with respect to the ethnicity also as these are the first breast cancer cell lines to be established from Indian continent representing early onset breast cancers. In conclusion, in the present study we have established two novel triple negative breast cancer cell lines, NIPBC-1 and NIPBC-2 from primary tumors of two early onset breast cancers (<40 years), which may serve as valuable *in vitro* models to study breast tumorigenesis in young breast cancer patients and identification of unique therapeutic targets.

Chapter 5

Isolation and Quantitation of Breast Cancer Stem Cells

Chapter 5: Isolation and Quantitation of Breast Cancer Stem Cells from Breast Cancer Cell Lines

5.1. Introduction: Studying cancer stem cell behavior is important in understanding cancer pathogenesis. It has been recently shown that human breast cancer stem cells (BCSCs) can be identified and sorted out based on the presence of aldehyde dehydrogenase (ALDH) marker. According to a report, the human genome contains 19 ALDH functional genes and three pseudogenes [322]. Many allelic variants within the ALDH gene family have been identified. Out of these ALDH1A1 enzyme has been studied extensively and is reported to play a pivotal role in the proliferation and drug resistance of cancer stem cells of various origins like breast, lung, prostate and heamatopoeitic progenitors [240, 241, 323, 324]. Al-Hajj and colleagues have demonstrated the presence of the ALDH-positive phenotype cells with stem cell properties in both normal and malignant breast samples [27]. This functionally distinct cell type can be identified by the expression of aldehyde dehydrogenase enzyme using flow cytometry and the ALDEFLUOR assay, which measures aldehyde dehydrogenase 1 class A1 (ALDH1A1) activity.

BCSCS can be further enriched and propagated in suspension cultures as 'mammospheres'. However, little is known about the behavior of these cells in long-term cultures. The inability to maintain cancer stem cells in an undifferentiated state in vitro has further marred their characterization. It was reported earlier that primary mammosphere-derived cells can initiate secondary mammospheres, as well as differentiate to give rise to various breast lineages, thereby exhibiting two fundamental properties of stem cells – self-renewal and multilineage differentiation. Additionally, both murine and human mammospheres have the potential to generate ductal-alveolar outgrowths in vivo, further demonstrating the existence of stem cells within these units [21][22]. Thus, the mammosphere system offers an in vitro model to study mammary stem cell biology and pathogenesis. Indeed, the involvement of Notch and Hedgehog signaling pathways which were reported to be derailed in breast cancer, in regulating mammary stem cell self-renewal has been demonstrated using the mammosphere system [22, 23]. However, little is known about the behavior of

breast stem cells or BCSCS within mammospheres in long-term cultures. Since extensive self-renewal potential is the hallmark of stem cells, we undertook a detailed functional characterization of human mammospheres over long-term passages.

According to a report, the human genome contains 19 ALDH functional genes and three pseudogenes [322]. Many allelic variants within the ALDH gene family have been identified. Out of these ALDH1A1 enzyme has been studied extensively and is reported to play a pivotal role in the proliferation and drug resistance of cancer stem cells of various origins like breast, lung, prostate and heamatopoeitic progenitors [240, 241, 323, 324]. However, the intricate mechanism of drug resistance is not well understood. Al-Hajj and colleagues have demonstrated the presence of the ALDH-positive phenotype cells with stem cell properties in both normal and malignant breast samples [27]. This functionally distinct cell type can be identified by the expression of aldehyde dehydrogenase enzyme using flow cytometry and the ALDEFLUOR assay, which measures aldehyde dehydrogenase 1 class A1 (ALDH1A1) activity. Targetting BCSCs by the inhibition and/or knockdown of stem cell markers like CD44, ALDH1 or ABCG2, resulting in the loss of stemness and an increase in the susceptibility to chemo/radio therapy offers a potentially promising and effective strategy for breast cancer treatment.

5.2. Materials and Methods:

5.2.1. Cell Lines and Reagents: Human breast cancer cell lines PBB20, PCB36, MCF7, T47D, SUM159, and MDA MB 453 along with human breast normal epithelial cell line HBL100 were used in this study. PCB20, PCB36 and MCF7 were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 1% Penstrep (Invitrogen, Carlsbad, CA), and 5 μg/ml insulin (Sigma-Aldrich, St Louis, MO). SUM159 was maintained in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum, 1% antibiotic-antimycotic, 5 μg/ml insulin and 1μg/ml hydrocortisone (Sigma- Aldrich, St Louis, MO). T47D, HBL100 and MDA MB453 cell lines were maintained in RPMI, McCoy and Leibovitz-15 media

respectively, supplemented with 10% FBS and 1% Penstrep. All the cell lines were maintained at 37 °C in 5% CO_2 saturated atmosphere except MDAMB453 cell line which does not require CO_2 for its proliferation. The list of breast cancer/normal cell lines used in this study along with their hormone receptor status are given in Table.1

5.2.2. Quantitation of Breast Cancer Stem Cells (BCSCs): A cell population with a high Aldehyde dehydrogenase (ALDH) enzyme activity was previously reported by Al-Hajj *et al.,* to enrich mammary stem/progenitor cells. Hence Breast Cancer Stem Cells (BCSCs) were quantitated and sorted out based on the presence of the aldehyde dehydrogenase (ALDH) marker using Aldefluor assay.

5.2.2.1. Aldefluor Assay and Flow Cytometry:

Aldefluor assay was performed as per the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada). Single cells obtained from cell cultures were incubated in Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde for 45 min at 37 °C. As a negative control, a fraction of cells from each sample were incubated under identical condition in the presence of ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Flow cytometry was conducted using FACS ARIA II SORP (Special order research product) (Becton Dickinson). ALDEFLUOR florescence was excited at 488nm and florescence emission was detected using a standard FITC 530/30 band pass filter. The sorting gates were established using the negative control.

5.2.3. Mammosphere Formation Assay: Mammospheres were grown in serum-free, low adherence cultures, as described by Dontu *et al*. The sorted out $ALDH^{+/bright}$ cells (termed as mammosphere forming Units – MFUs) and the ALDH-negative cells were plated in six-well ultra low attachment plates (Corning, MA) in triplicates for the formation of primary mammospheres at a density of $2.5x10^3$ cells/well. The resulting primary mammospheres after 10 days When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells (data not shown). Thus, we hypothesize that lack of mammosphere formation could not be attributed to the

absence of live cells beyond the sixth/seventh passage, but due to differentiation of cancer stem cells.

termed M1 mammospheres) were collected by centrifugation at 500 rpm for 03 mins. Mammospheres were counted microscopically under a manually prepared quadrant grid' and were dissociated both enzymatically and mechanically to obtain single cell suspension. The dissociated cells were sorted again using FACS. To test the functional definition of stem cells to self-renew, these MFUs enriched for breast stem and early progenitor cells were again plated after live cell count for mammosphere formation and were subjected to serial passaging every 10^{th} day leading to the generation of M2, M3, M4 mammospheres and so on. The mammospheres were counted after every passage and the images were recorded using ImagePro software.

5.3. Results:

5.3.1. Aldeflor assay and Flow Cytometry: All the breast cancer cell lines were grown as adherent cultures. The cells were harvested when they are in their log phase of growth and were stained with aldefluor reagent using aldefluor kit as per the manufacturer's instructions. The $ALDH^{+/bright}$ cells were then sorted out using FACS ARIA II. Representative pictures of the set of dot plots showing the percentage of ALDH-positive breast cancer stem cells in the parent population of both breast cancer/normal cell lines (Table 5.1) were shown in Figure 5.1 & 5.2. Analysis by flow cytometry revealed that MCF7, MDA MB 453, HBL100 and SUM159 breast cancer cell lines have 0.2%, 0.15%, 0.4%, and 0.7% ALDH-positive BCSCs respectively. Whereas T47D, PCB20 and PCB36 cell lines were found to possess no ALDH-positive BCSCs.

Cells obtained by the enzymatic dissociation of mammospheres at each passage were again sorted, and the ALDH-positive cells were further seeded under similar culture conditions for serial mammosphere assays (Figure 5.3. & 5.4). We have termed the mammospheres formed by plating the parent ALDH-positive population as 'M1', such that the subsequent passages of mammospheres after

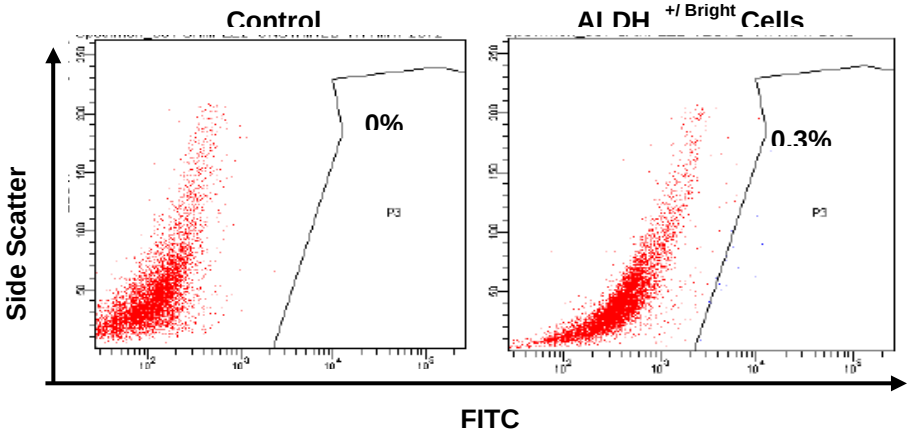
enzymatic dissociation were termed as M2, M3 and so on. The time interval between each passaging was nine days. Since mammosphere formation was not observed in the ALDH-negative fraction, these cells were not plated in the subsequent passages.

Cell lines	Molecular subtypes	Aldefluor phenotype	ER	PR	ERBB2	Source	Histological Type	Age
MCF7	Lu	0.2- 0.46%	+	+	-	PE	IDC	69
T-47D	Lu	0	+	+	-	PE	IDC	54
SUM159	Mes	0.7- 1.2%	-	-	-	PT	An Ca	-
HBL100	Ba.B	0.3%-0.4%	+	-	-	Breast milk	-	27
MDA MB 453	Lu	0.3%	-	-	+	PE	Ac	48
PCB20	Lu	0%	-	-	-	PT	IDC	39
PCB36	Lu	0%	-	-	-	PT	IDC	38

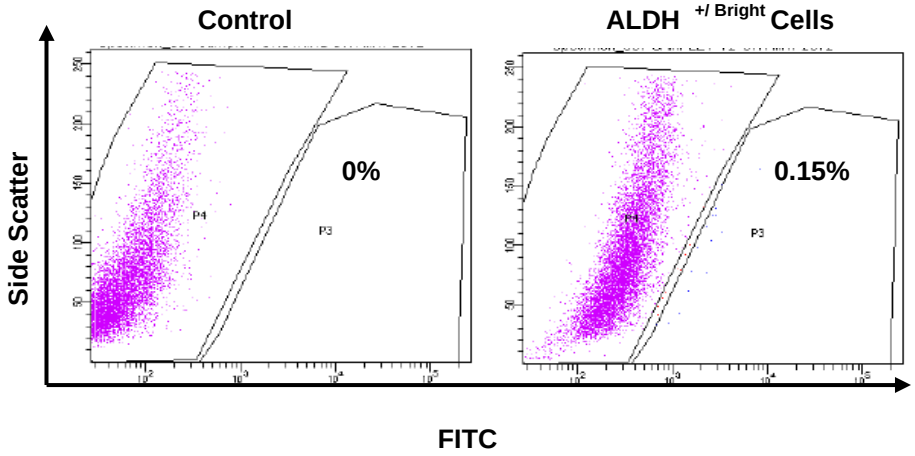
Table 5.1. List of Breast cancer/normal cell lines used in the present study along with their hormone receptor status

5.3.2. Mammosphere formation: Breast cancer cell lines contain distinct populations that display $ALDH^{-/low}$ and $ALDH^{+/bright}$ phenotypes. In order to find out which of these fractions housed cells capable of initiating mammospheres, we sorted out the same number of both ALDH-positive and ALDH-negative cells and assessed their sphere forming ability. In the present study, we found that $ALDH^{-/low}$ cells fail to generate mammospheres. Instead, the mammosphere–initiating potential rests within the $ALDH^{+/bright}$ cells, in keeping with the phenotype of breast cancer–initiating cells. ALDH-positive cells were seeded in low-attachment plates in media devoid of serum. Floating spheroids, termed mammospheres, were observed within 6- 8 days of plating.

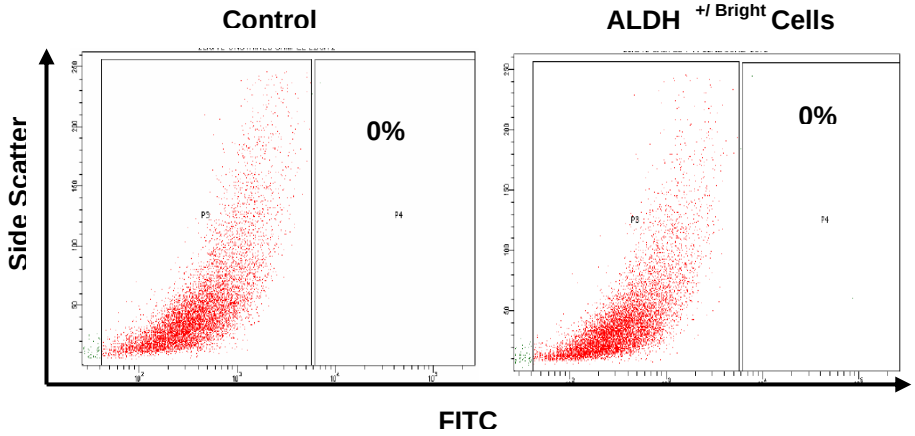
(a) MCF7



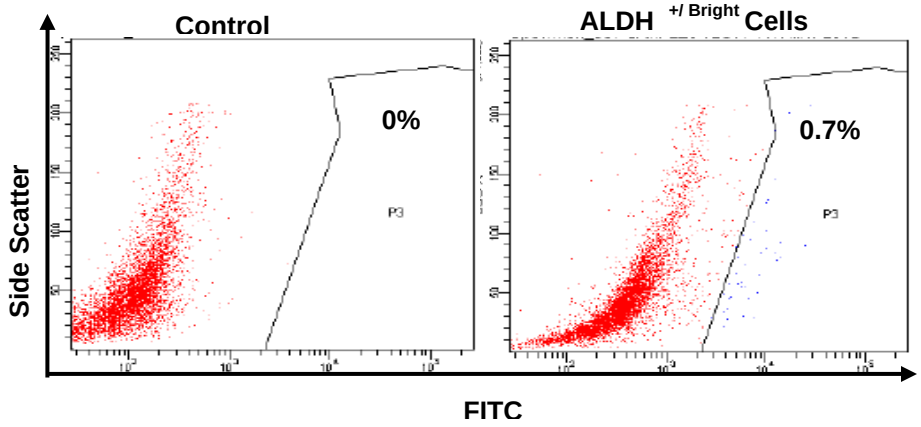
(b) MDA MB 453



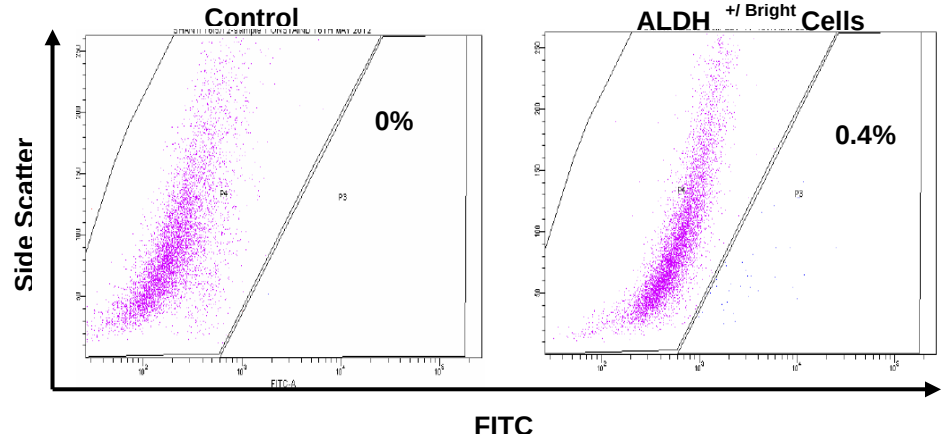
(c) T47D



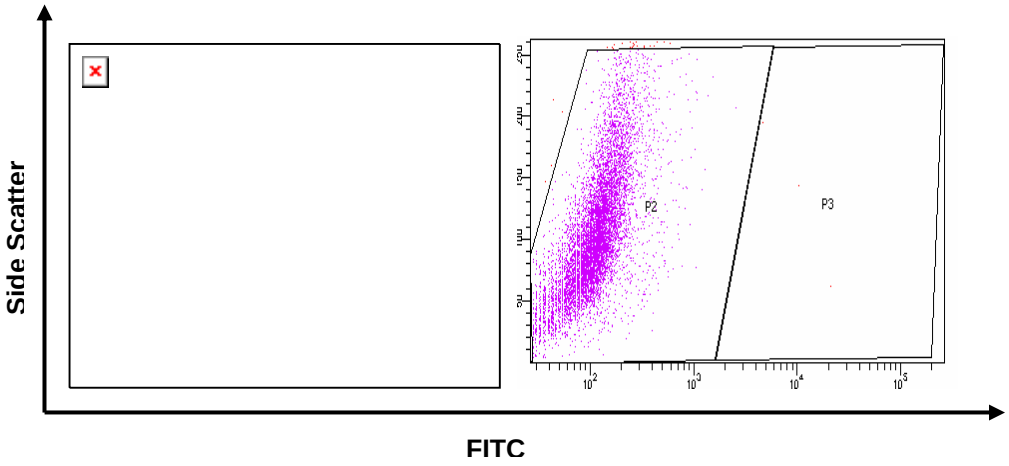
(d) SUM159



(e) HBL100



(f) PCB20



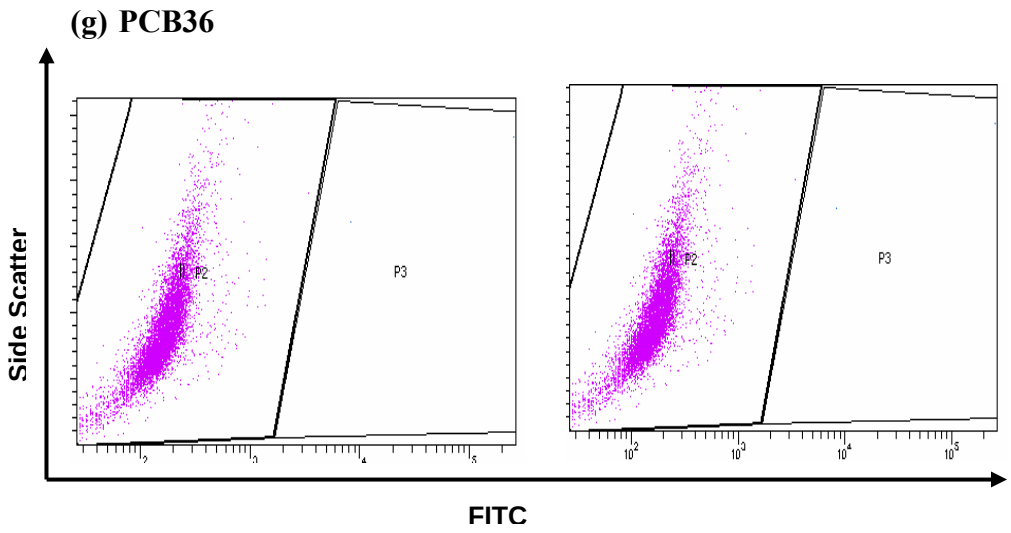


Figure 5.1. Representative pictures of the set of dot plots showing the percentage of ALDH-positive breast cancer stem cells in the parent population of breast cancer/ normal cell lines.

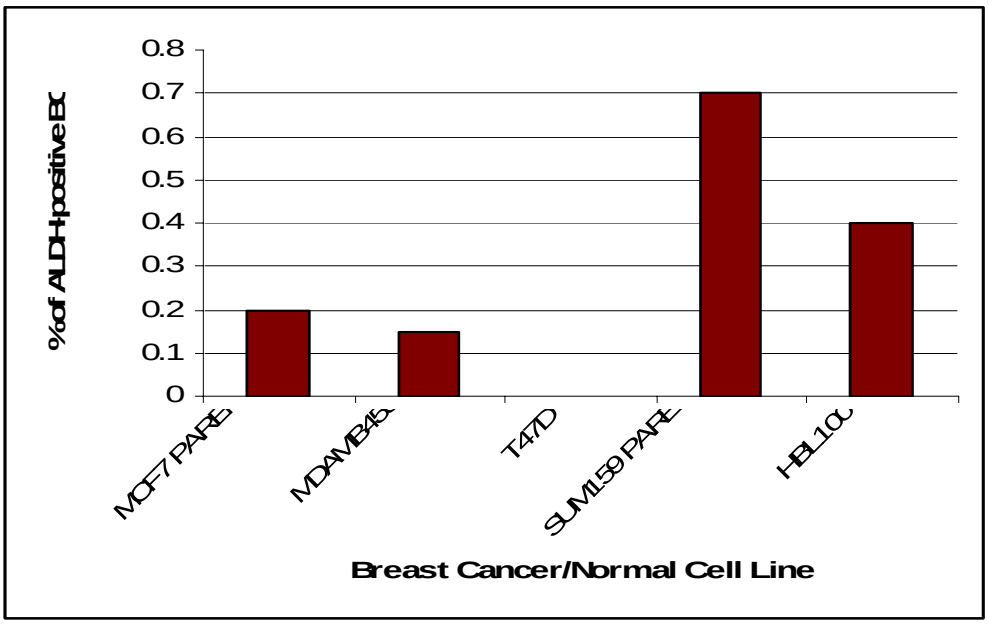
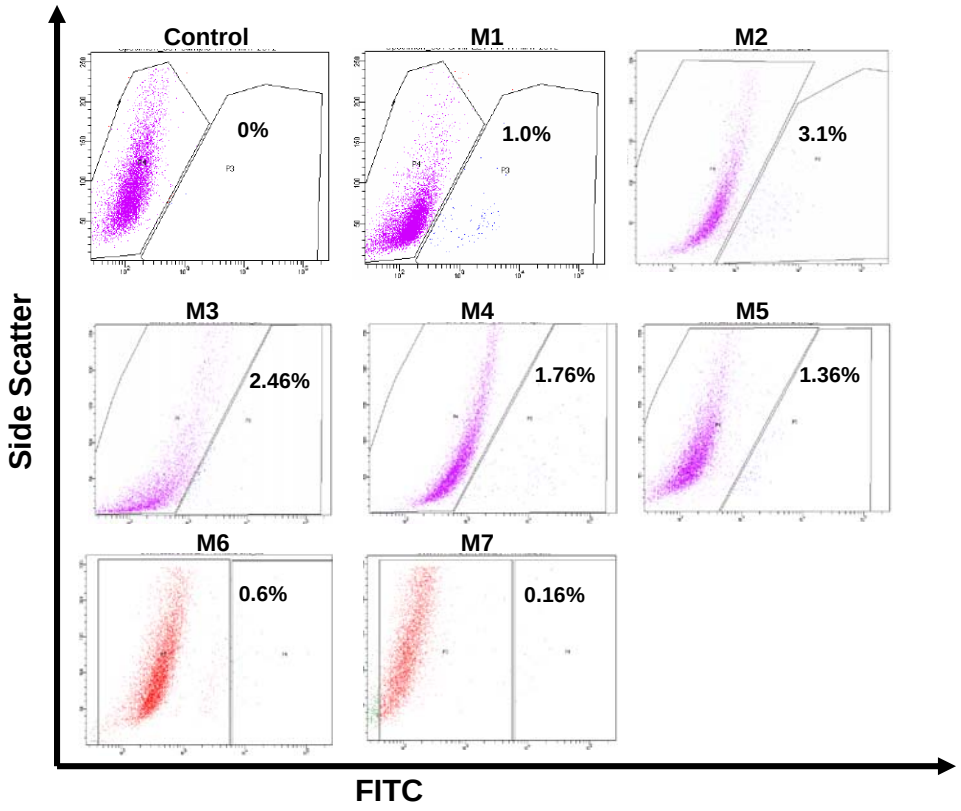


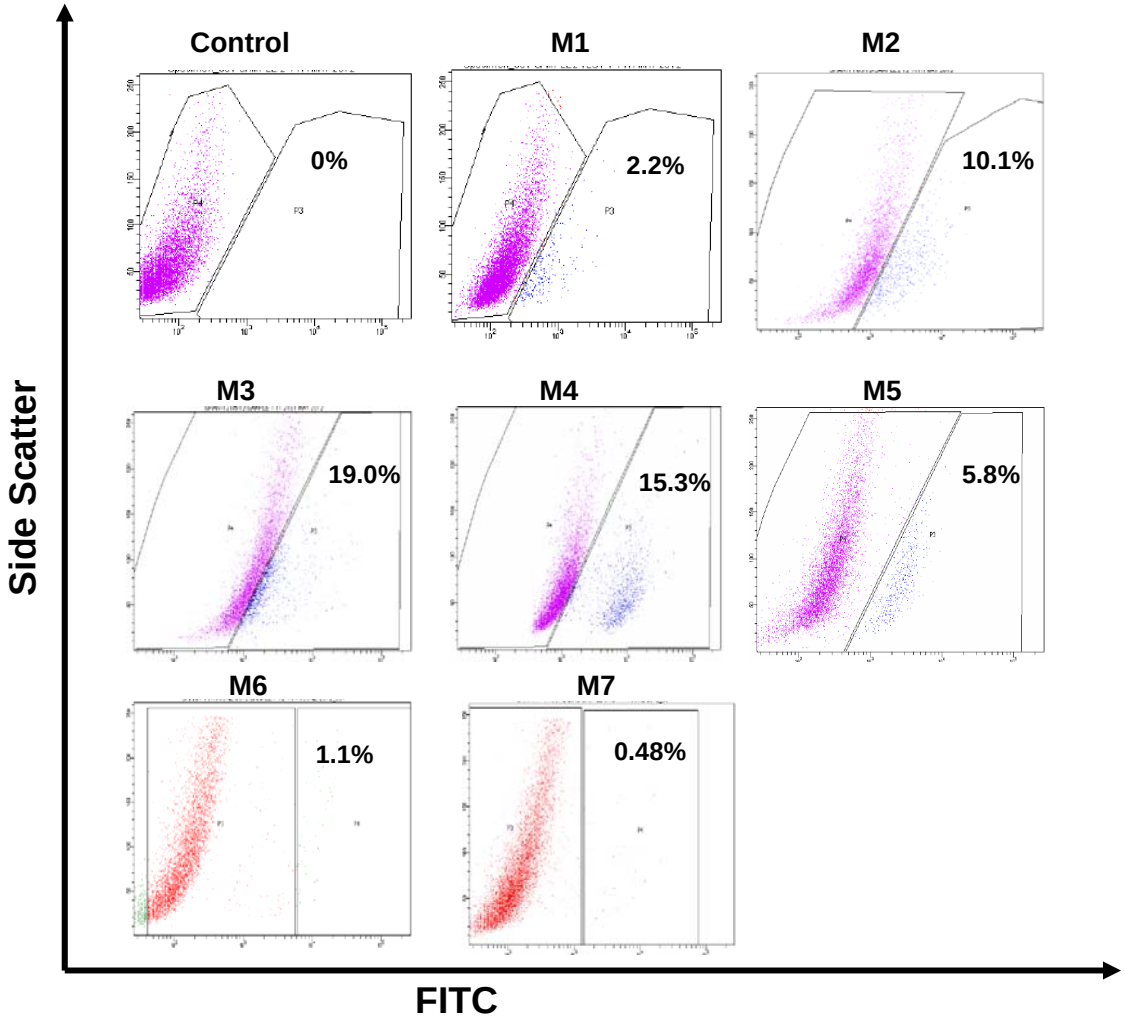
Figure 5.2. Graphical representation of the percentage of ALDH-positive breast cancer stem cells (BCSCs) present in the parent population of various breast cancer/ normal cell lines

5.3.3. Self Renewal ability assay: In the present study, we observed that with increasing passages, the MCF7 and HBL100 mammospheres showed a dynamic increase in the number of MFUs from ~0.2% to ~3.10% and ~0.4% to ~3.2% respectively until the second generation (M2) followed by a gradual reduction to 0.15% to 0.20% respectively by the seventh (M7) and sixth (M6) generations respectively. Whereas for SUM159 cell line, the percentage of MFUs increased from ~0.7% to ~10.10% until the third generation (M3) and declined subsequently to ~0.35% by the seventh generation (M7) (Figure 5.3 & 5.4). Surprisingly, the ALDH-positive cells derived from MDAMB453 cell line tend to form looser and lesser rounded mammospheres under non-serum, non-adherent conditions. This might indicate that the shape and appearance of mammospheres is cell line dependent as the other three cell lines ie., MCF7, SUM159 and HBL100 used in this study formed rounded mammospheres under similar non-serum, non-adherent conditions (Figure 5.5).

(a) MCF7



(b) SUM159


Control
M1
M2
0%
2.2%
10.1%
M3
M4
M5
19.0%
15.3%
5.8%
M6
M7
1.1%
0.48%
Side Scatter
FITC

(c) HBL100

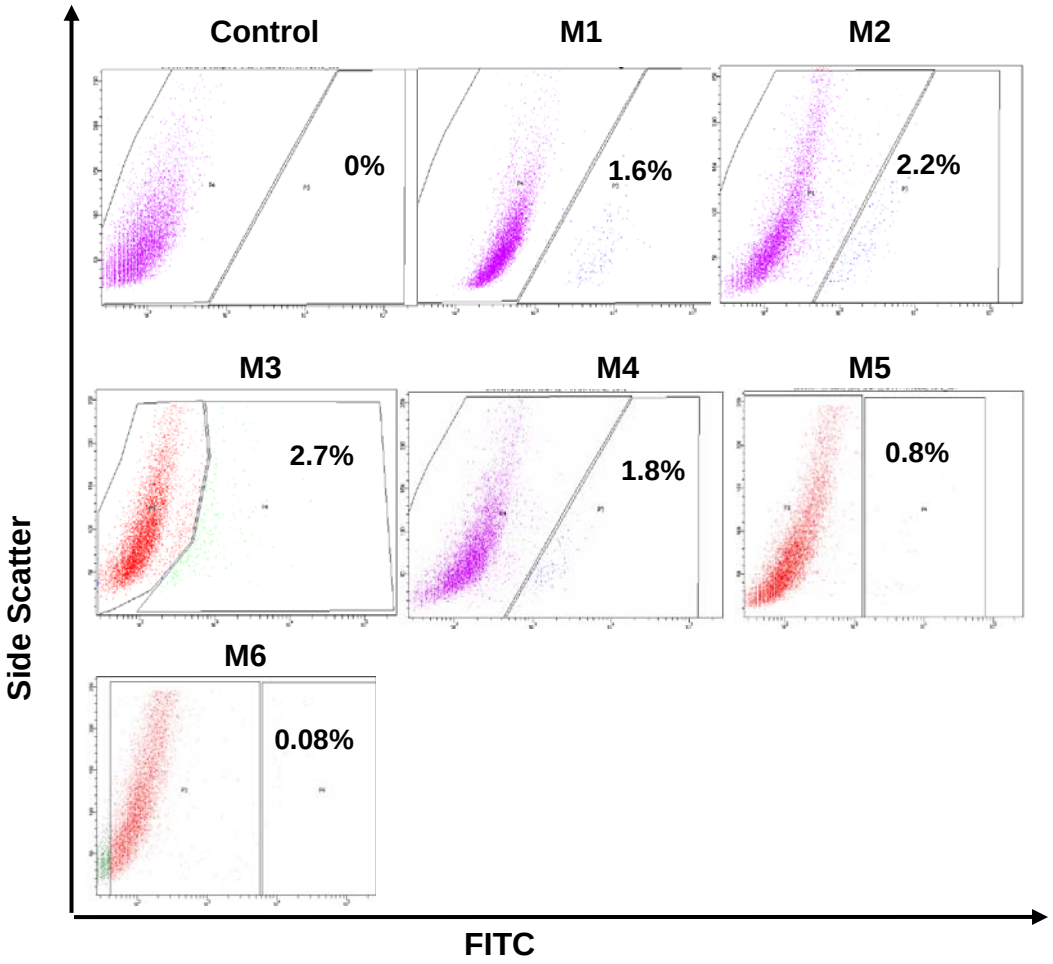


Figure 5.3. Representative pictures of the set of dot plots showing the percentage of ALDH-positive breast [50] stem cells after serial passaging of mammospheres derived from both breast cancer/normal cell lines. Gating was done according to the individual DEAB controls.

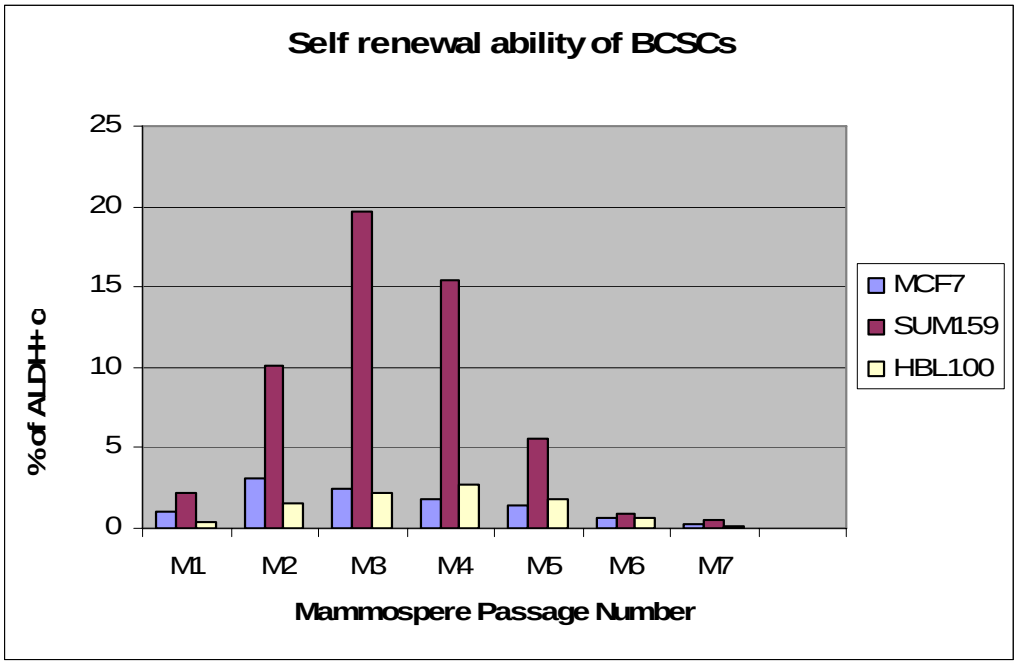
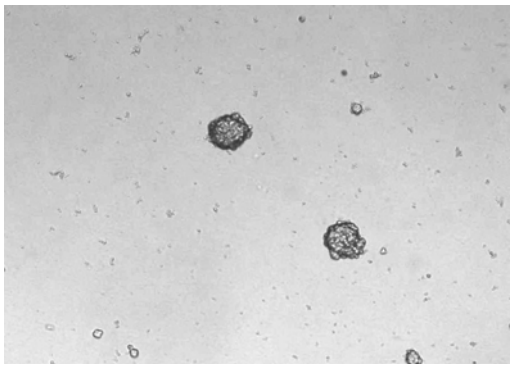


Figure 5.4. Graphical representation of the percentage of ALDH-positive breast cancer stem cells (BCSCs) after serial passaging of mammospheres, which depicts the self-renewal ability of mammospheres derived from various breast cancer/ normal cell lines as analysed by aldefluor assay.

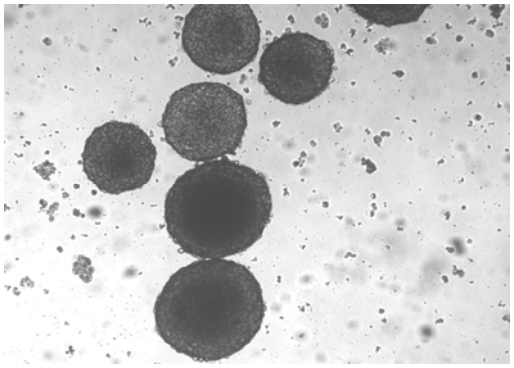
5.4. Discussion: Sphere formation efficiency at each passage was calculated by dividing the total number of spheres formed by the total number of live cells seeded multiplied by hundred. We observed an increase in sphere forming efficiency from M1 to M2 (from 10.4% to 31.66%) in SUM159 cell line whereas it is from M1 to M3 in both MCF7 (5.8% to 25.8%) and HBL100 (4.9% to 23.8%) cell lines , but a decline thereafter among all the three cell lines (Figure 5.6). The mammospheres obtained after 10 days ranged in size from ~510µm in diameter, with the majority of spheres having a diameter of ~340µm. On analyzing the size of mammospheres through subsequent passages, we found that the size of the largest and smallest spheres in each passage remained almost consistent; however, the relative proportion of smaller spheres increased in number with passage (Figure 5.7). Interestingly, we failed to detect mammosphere formation beyond the seventh passage in both MCF7 and SUM159 cell lines and beyond the sixth passage in the breast normal epithelial cell line after which, mostly single cells and few clumps/aggregates of cells were seen.

(a) MCF7

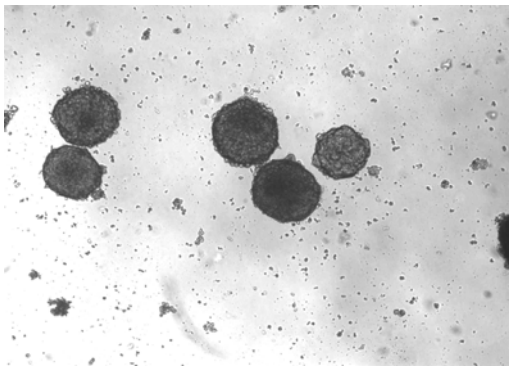
M1



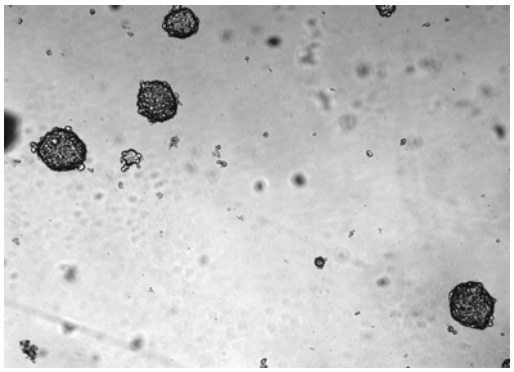
M2



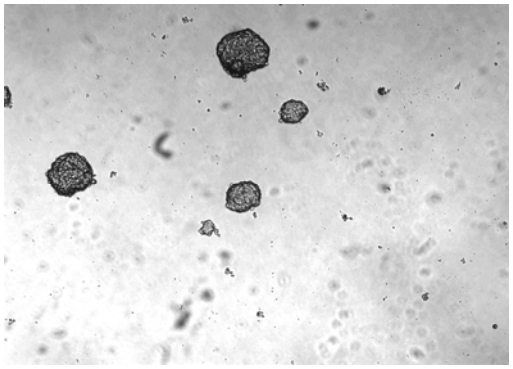
M3



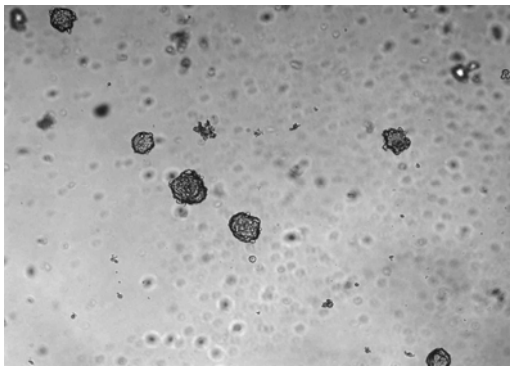
M4



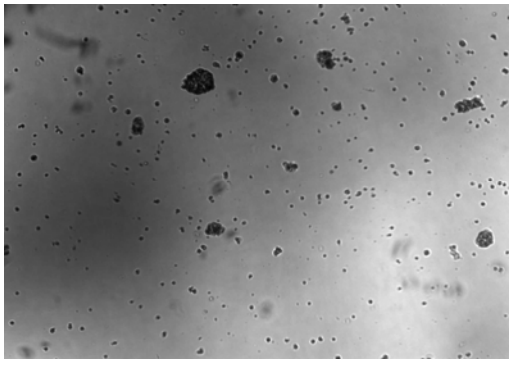
M5



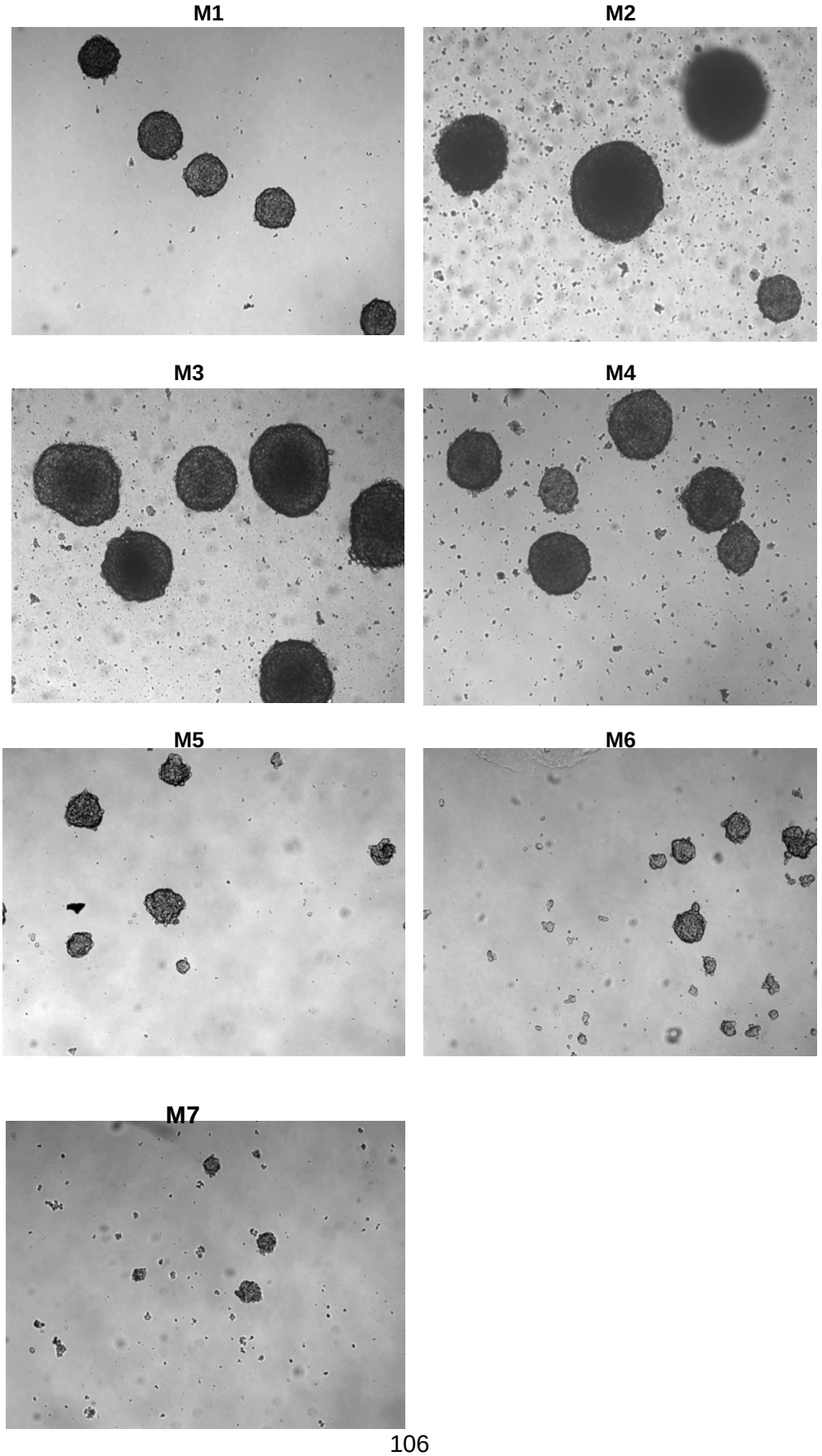
M6



M7

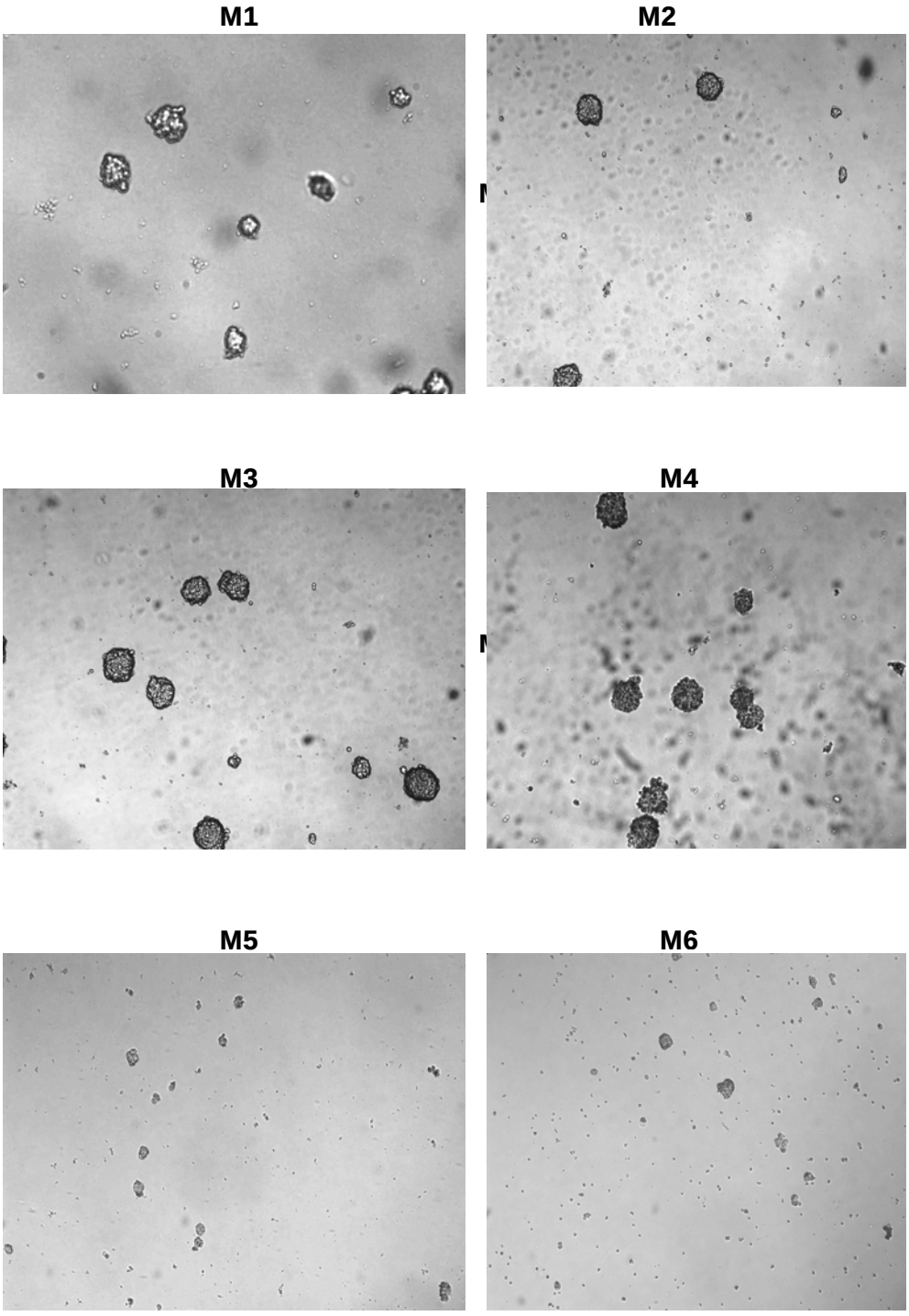


(b) SUM159



M6

(c) HBL100



(d) MDA MB 453

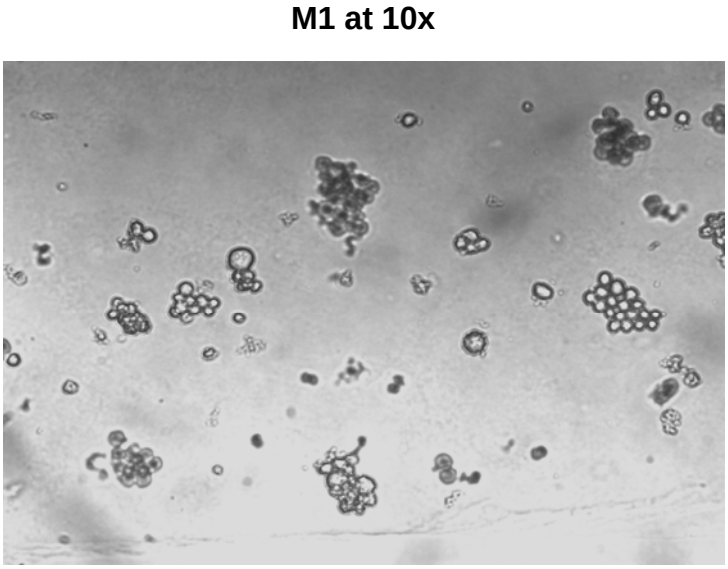


Figure 5.5. Representative pictures of mammospheres formed by various breast cancer/ normal cell lines after serial passaging. The mammosphere images of all the cell lines were captured using ImagePro software with a Nikon microscope at 4x magnification unless otherwise mentioned.

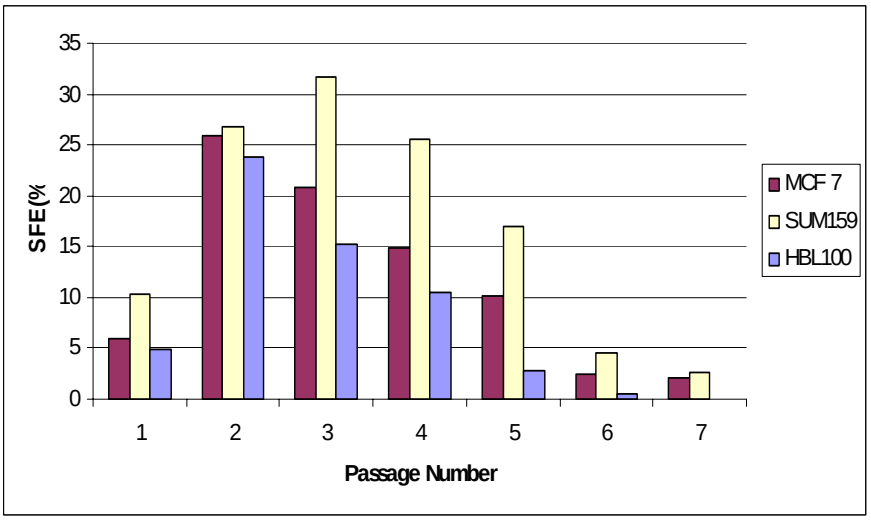


Figure 5.6. Sphere Formation Efficiency (SFE) of various breast cancer/ normal cell lines.

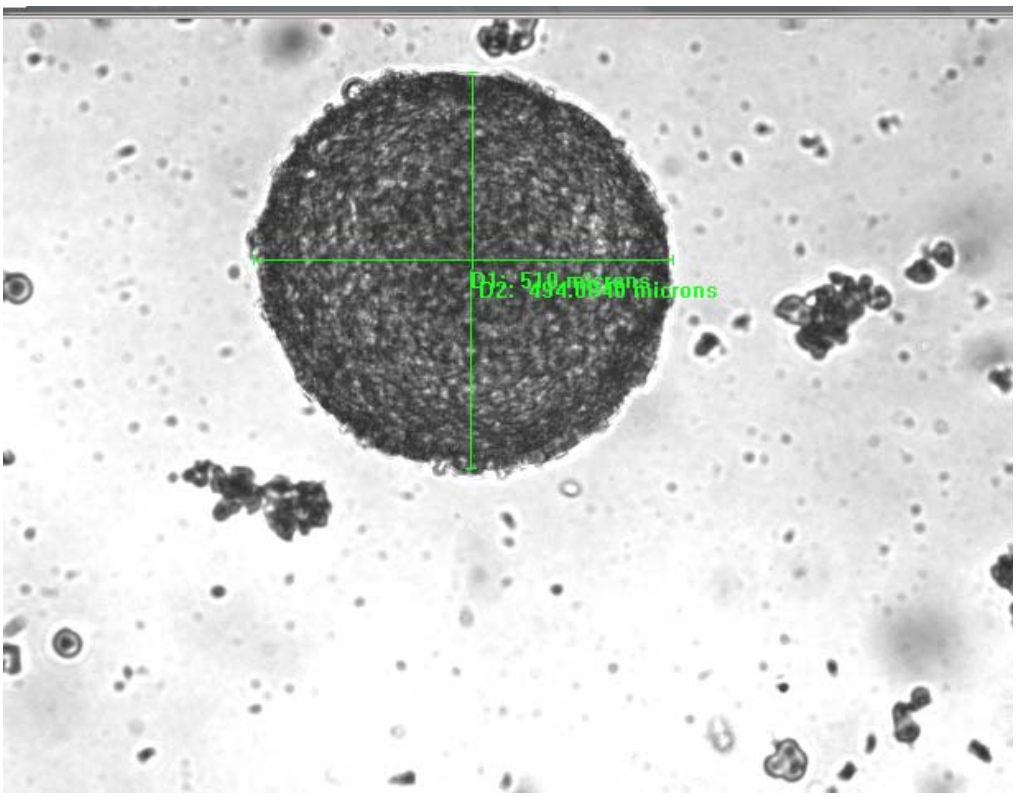


Figure 5.7. Representative picture showing the size of a mammosphere

When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells (data not shown). Thus, we hypothesize that lack of mammosphere formation could not be attributed to the absence of live cells beyond the sixth/seventh passage, but due to differentiation of cancer stem cells.

In this study, we show that the sphere forming and self-renewal potential resides within the ALDH-positive population of 'aldefluor' stained mammospheres. In serial sphere formation assays, we found variations both in the size and number of mammospheres over passages. Importantly, we fail to detect generation of new mammospheres beyond the seventh passage despite the presence of live cells.

Chapter 6

Study of Effect of Anti Cancer Drugs on Breast Cancer Stem Cells

Chapter 6: Effect of anticancer drugs on Breast Cancer Cell Lines with High Proportion of Stem cells as Compared to those with Low Proportion of Stem Cells

6.1. Introduction

Breast cancer is the second most leading cause of cancer deaths in India accounting for 24 % next to cervical cancer, as per the NCRP (National Cancer Registry Program) data [325]. There has been significant rise in the incidence of breast cancer during last two decades, showing the magnitude of the current health problem associated with breast cancer in Indian population.

Tumor recurrence and treatment failure are two well known factors in cancer therapy associated with poor survival and recently they have been linked with cancer stem cells (CSCs) [19, 20]. The CSCs may not respond to primary treatment similar to bulk cancer cells, resulting in resistance and relapse [326-330]. Hence, identification of chemosensitizers for cancer therapy is an area of intense investigation. Breast cancer stem cells (BCSCs) are increasingly thought to play a major role in breast cancer growth, relapse and metastasis. They are characterized by the combined expression of cell surface markers $CD44^{+}/CD24^{-}$ and by their ability to pump out dyes like Hoechst (Side population cells) [27]. It has been recently identified that BCSCs can also be identified and sorted out based on the presence of aldehyde dehydrogenase (ALDH) enzyme [284, 331, 332]. Though BCSCs have been identified in many organs like breast, lung, prostate and heamatopoeitic progenitors their intricate mechanism of drug resistance is not well understood. Targetting BCSCs by the inhibition and/or knockdown of stem cell markers like CD44, ALDH1 or ABCG2, resulting in the loss of stemness and an increase in the susceptibility to chemo/radio therapy offers a potentially promising and effective strategy for breast cancer treatment.

The anti neoplastic alkaloid Ellipticine (5,11-dimethyl-6H-pyrido [4,3-b] carbazole), found in Apocyanaceae plants eg., Ochrosia borbonica, O.elliptica, is a prodrug [333]. Cytochrome P450 enzymes oxidize ellipticine, forming up to five metabolites viz., 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine, and

ellipticine N_2-oxide. Out of these, 9-hydroxy- and 7-hydroxyellipticine are the major detoxification products of ellipticine oxidation *in vivo*. It's pharmacological efficiency is dependent on its cytochrome P450 (CYP)- and/or peroxidase-mediated activation in target tissues [334, 335]. In human breast adenocarcinoma MCF-7 cells, ellipticine causes arrest of G2/M phase of the cell cycle associated with an increased expression of p53, p21/ WAF1, and KIP1/p27 proteins, and growth inhibition induced by mitochondrial proapoptotic pathways [336]. In addition, several derivatives of ellipticine have been demonstrated to be active against brain tumor cell lines and have shown promising results in treatment of metastatic breast cancer [337, 338]. P53 mutations occur in over half of all human tumors and serve as unique molecular targets for cancer therapy. Recent advances demonstrate that reconstitution of p53 function is possible and practical as a promising anti tumor therapy. The P53 restoration activity along with the complete lack of hematological and hepatic toxicity by ellipticine and its derivates [339] present it to be an effective anti-cancer agent.

The P53 restoration activity of ellipticine has been demonstrated in colon cancer stem cells in a very recent study [340]. In the present study we examined the effect of ellipticine on breast cancer stem cells expressing ALDH1A1 marker *in vitro* in comparison with paclitaxel which is administered in conventional chemotherapy. We also demonstrated the effect of ellipticine on mammosphere formation and self- renewal ability of BCSCs in combination with paclitaxel. Further, we also evaluated the efficacy of ellipticine on the activity of human ALDH1A1 by molecular docking of ellipticine and its derivatives with the energy minimized model of Homo sapiens ALDH1A1 preceded by *in silico* homology modeling of the enzyme.

6.2. Materials and Methods

6.2.1 *In vitro* studies

6.2.1.1. Cell Lines and Reagents

Human breast cancer cell line MCF7 was obtained from American Type Culture Collection and SUM159 was a kind gift from Dr. Madhuri Kakarala, University of

Michigan. MCF7 is derived from the pleural effusion of metastatic breast carcinoma and possess a wild type p53. This cell line is ER and PR positive and Her2μ negative. SUM159 is a p53 mutant cell line derived from primary breast anaplastic carcinoma. This cell line is ER negative, PR negative, and does not have Her2μ over-expression. Both the cell lines were tested and were found to be free of mycoplasma contamination. They were maintained carefully in culture as described below. MCF7 was maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 1% Penstrep (Invitrogen, Carlsbad, CA), and 5 μg/ml insulin (Sigma-Aldrich, St Louis, MO). SUM159 was maintained in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum, 1% antibiotic-antimycotic, 5 μg/ml insulin and 1μg/ml hydrocortisone (Sigma- Aldrich, St Louis, MO). Ellipticine, paclitaxel and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St Louis, MO).

6.2.1.2. Aldefluor Assay and Flow Cytometry

A cell population with a high Aldehyde dehydrogenase (ALDH) enzyme activity was previously reported by Emmanuelle Charafe-Jauffret and colleagues to enrich mammary stem/progenitor cells. Aldefluor assay was performed as per the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada). Single cells obtained from cell cultures were incubated in Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (BAAA, 1μmol/L per 1,000,000 cells), for 30 min at 37 °C. As a negative control, a fraction of cells from each sample was incubated under identical condition in the presence of ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Flow cytometry was conducted using FACS ARIA II SORP (Special order research product) (Becton Dickinson). ALDEFLUOR florescence was excited at 488nm and florescence emission was detected using a standard FITC 530/30 band pass filter. The sorting gates were established using the negative control.

6.2.1.3. MTT Cell Proliferation Assay

The cytotoxicity of ellipticine and paclitaxel was determined by MTT test. Both MCF7 and SUM159 cells present in their exponential growth phase were seeded at 1×10^4 cells per well in a 96-well plate (Corning). For a dose-response curve,

DMSO stock solutions of ellipticine (10 mM) and paclitaxel (10 μM) were dissolved in culture medium to get final concentrations of 0.5–8 μM for ellipticine and 0.5-8nM for paclitaxel. The cells of both the cell lines were replenished with fresh media containing varying concentrations of both the drugs on the next day and incubated for 48 hours at 37 °C in 5% CO_2 saturated atmosphere. Wells containing only the cells and medium with no drug added, were taken as controls. After incubation the cells were fed with 200μl of fresh medium, 50μl of MTT (Sigma) solution (2 mg/ml PBS) was added and the plates were incubated for 3 hours. After incubation the media containing MTT solution was removed and the formazan crystals were dissolved by adding 200μl of DMSO to each well. The absorbance was read at 570nm for each well immediately using a multiwell spectrophotometer. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviation.

The ALDH-negative cells of both the cell lines, which were sorted after aldefluor assay were also seeded at a density of 1×10^4 cells per well in a 96-well plate and were treated as described above for the MTT assay and the IC_{50} values were calculated.

6.2.1.4. Effect of ellipticine and paclitaxel on ALDH1A1 –positive cells in breast cancer cell lines

Both the cancer cell lines were seeded as adherent cultures at about 50-60% confluency overnight in 6-well plates (Corning) in triplicates. The next day, media was replaced with media containing ellipticine (2μM and 3μM), paclitaxel (2nM and 3nM) and DMSO to appropriate plates. The drugs were added both individually and in combinations as indicated in the figure 1. Also wells containing only media but not drugs or DMSO were taken as 'untreated cells' or 'parent' population. The cells were then incubated for 48 hours at 37°C in 5% CO_2 saturated atmosphere, washed with 1x PBS and trypsinised to get a single cell suspension. They were then resuspended in aldefluor buffer, stained with aldefluor substrate and assayed as mentioned above. A fraction of cells from each sample were incubated under identical conditions in the presence of DEAB and were used as negative control to establish gating.

6.2.1.5. Effect of ellipticine and paclitaxel on Mammosphere Formation

Mammospheres were grown in serum-free, low adherence cultures, as described by Dontu *et al* [26, 44]. Briefly, cell lines in their log phase of growth were dissociated both enzymatically and mechanically to get a single cell suspension and the ALDH1A1 positive cells were sorted using FACS after staining with aldefluor as mentioned above. The cells were then suspended in serum free media containing varying concentrations of both ellipticine (2μM and 3μM) and paclitaxel (2nM and 3nM) both individually and in combinations as indicated. They were then plated in six-well ultra low attachment plates (Corning, MA) in triplicates for the formation of primary mammospheres at a density of $1.5x10^3$ cells/well. The mammospheres were counted after seven days and the images were recorded using ImagePro software. The mammosphere numbers formed by drug(s) treated cells were normalized to control and plotted against drug concentration.

6.2.1.6. Ellipticine and paclitaxel's effect on the self renewal ability of mammospheres

Self-renewal capacity of the CSCs was determined by producing further generations of mammospheres. At each step, the mammospheres were dissociated to single cells, and replated under drug free low-adherence conditions. Briefly, mammospheres were collected and centrifuged at 300rpm for 5 minutes. After careful aspiration of the supernatant, 2ml accutase (Invitrogen, Carlsbad, CA) was added to the respective cell pellets and incubated for 2~4 mins at 37^0C. The mammospheres were then dissociated mechanically with a pasteur pipette, passed through a 45mm sieve (Becton Dickenson), counted and replated as mentioned above in a drug free medium. The size and numbers of mammospheres were quantitated and images were recorded using ImagePro software.

6.2.1.7. Statistical Analysis

Statistical differences were determined using two-tailed student t-test. Data are presented as mean ± SD ($n \geq 3$).

6.2.2. *In silico* studies

All the calculations were performed on a workstation AMD Opteron Duo-core 2.0 GHz and 4 GB RAM. Molecular modeling tasks were performed with Modeller9v11, Molecular dynamics calculation was carried out with Gromacs 4.5.3, docking calculations were performed with AutoDock 4.0. If not otherwise stated, default settings were used during all calculations.

6.2.2.1. Sequence alignment

The sequence of human ALDH1A1 (GI: 40807656) was obtained from the National Centre for Biotechnology Information (NCBI). Local alignments were predicted using Blastp (Basic Local Alignment Search Tool) [341] at the NCBI and the homologous entries were obtained from the protein data bank [342]. The Blastp alignment was further refined using sequence alignments in the clustalW 2.0 with default parameters [343].

6.2.2.2. Molecular model Building

The above alignment was used for comparative modeling built in the Modeller9v11 [344] which generated structures by applying spatial restraints. A bundle of 100 models from random generation of the starting structure was calculated and subsequently the best model with the low RMS value of superposition using Swiss-pdb viewer [345] was chosen for further analysis.

6.2.2.3. Validation of the homology modeled structure

To gain better relaxation and more correct arrangement of the atoms, refinement was done on the built human ALDH1A1 model by energy minimization (EM). The stereochemical parameters of the energy minimized ALDH1A1 model were assessed by Procheck [346], Whatif [347] ProSA [348] and Verify 3D [349, 350]. Verify 3D was used to assess whether a primary sequence is compatible with the current 3D structural model. Secondary structural conformations for the developed human ALDH1A1 model were predicted by pdbsum [351]. The developed 3D model of Human ALDH1A1 was submitted to Protein Model Data Base (PMDB), which collects 3D models obtained by structure prediction methods.

6.2.2.4. Molecular dynamics

Molecular dynamics (MD) calculation was carried out with Gromacs 4.5.3 [352], using Gromacs 96.1 (43 A^2)force field. All MD runs used a time step of 10 ns along with the LINCS routine algorithm to constrain bond lengths and SETTLE to constrain water geometry. The simulation was completed on 64 bit Athalon Intel 2.8 GHz AMD CPU. Built human ALDH1A1 model was immersed in a solvent octahedral box of 17413 SPC water molecules [353] (15 ˚A thick layer of water, with box size of 90 ˚Ax90 ˚Ax90 ˚A). Five Na+ counter ions were added to neutralize the system. A twin-range cutoff was used for log-range interactions: 1.5 nm for electrostatic interactions and 1.2 nm for Vander Waals interactions. The energy of the system was minimized to about 1000 cycles, using the steepest descent algorithm followed by heating to 300 K.

6.2.2.5. Docking analysis

Since ellipticine is a prodrug, we performed the docking studies for ellipticine, its metabolically active compounds- 12 and 13- hydroxyl ellipticine, its excretory products- 7 and 9- hydroxyl ellipticine and its peroxidase product N_2-Oxide ellipticine with structurally refined ALDH1A1. Based on the principles of empirical free energy function and Lamarkian genetic algorithm, Autodock 4.0 program is used to investigate the interaction between drug compound ellipticine and structurally refined ALDH1A1 model, in Grid Module by creating grid maps with 80 X 80 X 80 points and a grid-point spacing of 0.0375 nm. The grid maps representing the proteins in the actual docking process were calculated with AutoGrid. The grids (one for each atom type in the drug compound plus one for electrostatic interactions) were chosen to be sufficiently large to include not only active site but also significant portions of the surrounding surface. The settings of parameters were as follows: population size of 150, a maximum number of 25 million energy evaluations, a maximum number of generations of 27,000. The search was based on the Lamarckian genetic algorithm and the results were analyzed using binding energy. All torsion angles for ellipticine were considered flexible. A docking experiment was performed and the docking results were clustered according to root mean square deviation (RMSD) criterion of 0.1 nm and evaluated depending on the binding energy. After docking, the ligand-receptor

complexes were analyzed by Pymol program [354]. Molecular graphics were created using Pymol 0.99 (http://pymol.sourceforge.net).

6.3. Results

6.3.1. *In vitro* studies

6.3.1.1. Ellipticine and paclitaxel inhibit proliferation of Breast Cancer Cells

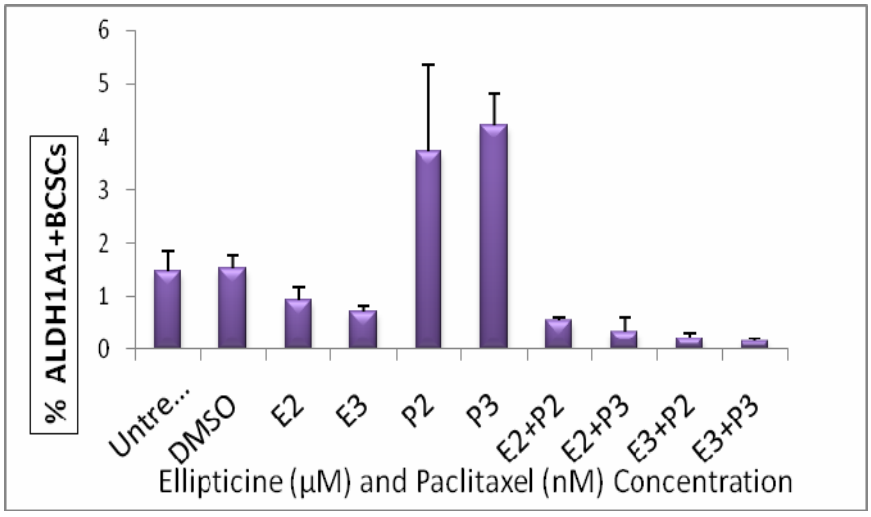
Both ellipticine and paclitaxel were previously shown to inhibit proliferation and induce apoptosis in breast cancer cells [355]. We evaluated the anti proliferative effects of ellipticine and paclitaxel in two breast cancer cell lines, MCF7 and SUM159 by MTT assay. Cells in their log phase of growth were treated with increasing concentrations of ellipticine and paclitaxel for 48 hrs. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Both the drugs decreased the viability of cells in a dose dependent manner. The IC_{50} of both the adherent bulk cells and ALDH-negative cells of MCF7 and SUM159 for ellipticine were found to be 5µM and 5.25µM respectively and the IC_{50} of paclitaxel were found to be 2.5nM and 3nM respectively. No difference in the IC_{50} values has been observed in the ALDH-negative cells as compared to bulk cancer cells of both the cell lines.

6.3.1.2. Ellipticine decreases the expression of ALDH1A1-positive BCSCs in both MCF7 and SUM159 cells

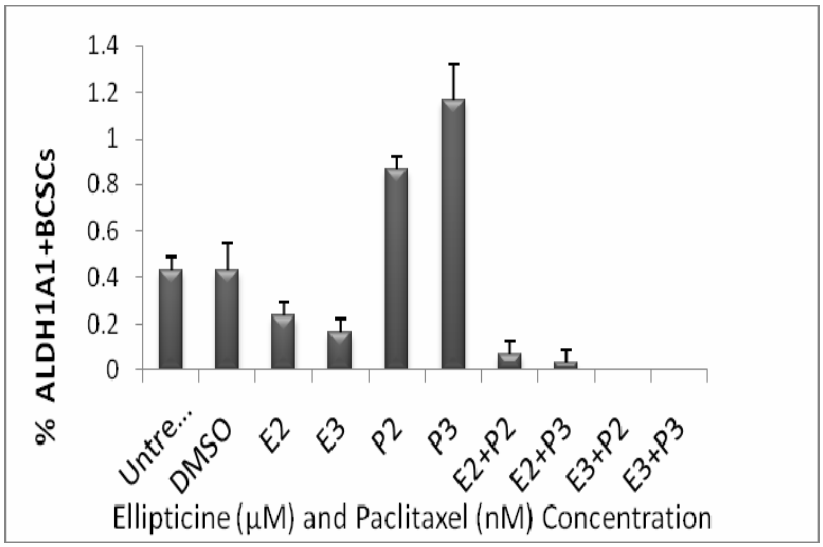
A decrease in the expression of ALDH1A1-positive breast cancer cells by 62% (p=0.073) in MCF7 cell line and by 53% (p= 0.024) in SUM159 cell line was found by ellipticine at 3µM concentration. On contrary, paclitaxel at 2 nM concentration showed increase in the percentage of ALDH1A1-positive breast cancer stem cells by 1.8 fold (p= 0.012) in MCF7 and by 2.4 fold (p= 0.008) in SUM159 cell line. Further, increasing the concentration of paclitaxel to 3nM, enhanced its ALDH1A1 enriching effect by 2.7 (p= 0.003) and 2.8 fold (p= 0.004) in MCF7 and SUM159 cell lines respectively compared to vehicle treated cells (Figure 6.1). This is in agreement with many of the previous observations which demonstrated that the use of conventional chemotherapy could lead to the enrichment of CSCs in cell lines, xenografted mice as well as in treated patients [253, 254]. Interestingly, the percentage of ALDH1A1-positive cells dropped by

7.5 fold (p= 0.008) and 11.5 fold (p= 0.006), in cells treated with a combination of 3µM ellipticine+2nM paclitaxel and 3µM ellipticine+3nM paclitaxel respectively in SUM159 cell line whereas a complete loss of ALDH1A1 marker expression was observed in MCF7 cells when treated with the above combinations, relative to the vehicle treated cells. Hence, increase in the concentration of paclitaxel reduced the expression of ALDH1A1-positive BCSCs when used in combination with ellipticine (Figure 6.1). This might suggest that, ellipticine has the ability to sensitize cells to conventional chemotherapeutic agents like paclitaxel leading to reduction in the expression of ALDH1A1-positive BCSCs.

(a)



(b)



(c)

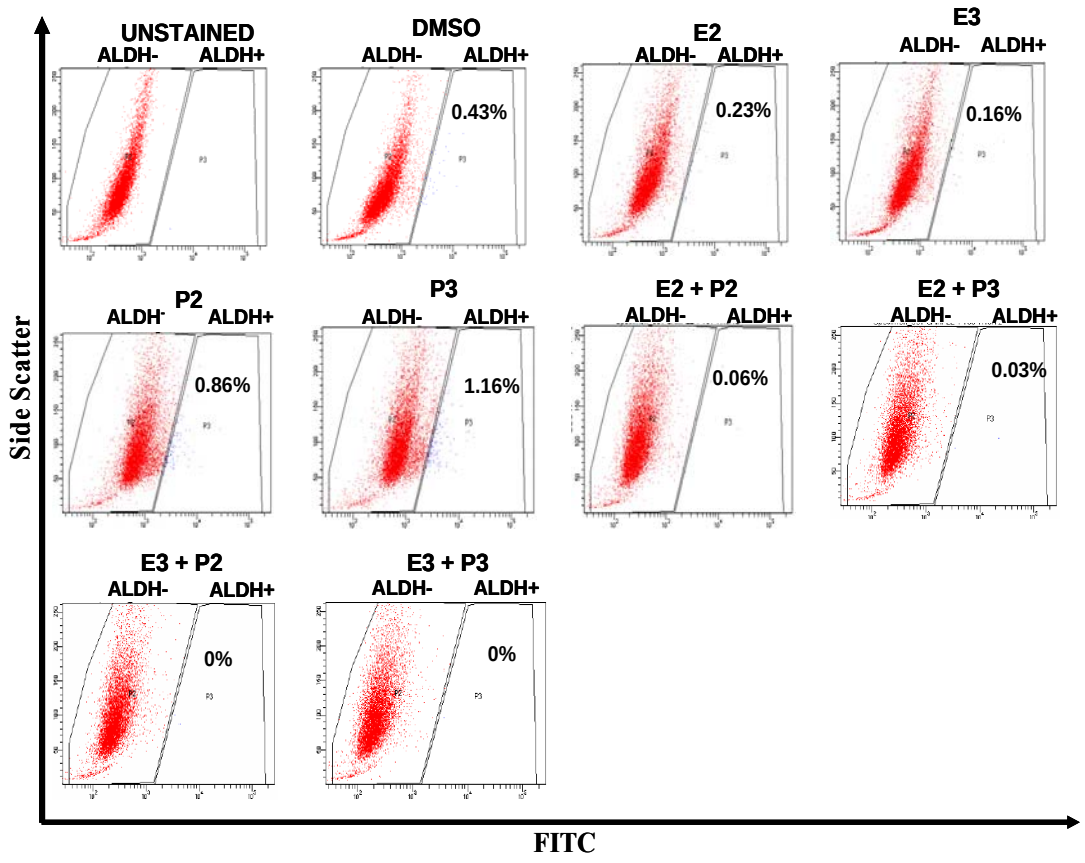


Figure 6.1. Depletion of MCF7 and SUM159 ALDH1A1-positive BCSCs after treatment with ellipticine in combination with paclitaxel contrasts with enrichment of BCSCs after treatment with paclitaxel alone. Both the cell lines were exposed to ellipticine (in µM) and paclitaxel (in nM) with the dosages indicated for 48 hours under adherent conditions and were analyzed by flow cytometry for the presence of ALDH1A1-positive BCSCs. (a) Representative data of experiment done in triplicates showing the percentage of ALDH1A1-positive BCSCs after drug exposure in both MCF7 and (b) SUM159 cell lines. (c) Representative pictures of expression profiles of ALDH-positive BCSCs after drug treatment in MCF7 cell line as analyzed by flow cytometry

6.3.1.3. Ellipticine inhibits mammosphere formation and reduced the self renewal ability of BCSCs *in vitro*

It was reported earlier that under non adherent conditions, stem cells and tumor initiating cells (TICs) form tumorospheres, whereas more differentiated cells tend to die by anoikis [45]. Previous studies have demonstrated that the ability of mammospheres to be serially passaged at clonal density is an indirect marker of stem cell self renewal [44]. Ellipticine significantly reduced the formation of mammospheres in a dose dependent manner relative to the vehicle treated cultures whereas paclitaxel enhanced mammosphere formation in both the treated cell lines (Figure 6.2). Treatment with 3µM ellipticine not only reduced the mammosphere numbers by 59% (p=0.012) and 65% (p= 0.002) in MCF7 and SUM159 cell lines respectively, but also a significant reduction in the size of the mammospheres was observed; whereas exposure to 3nM paclitaxel enhanced the mammosphere number by 1.3 (p= 0.019) and 1.4 fold (p= 0.003) in MCF7 and SUM159 cell lines respectively, with no significant change in the size (data not shown). Exposure of cells to a combination of 3µM ellipticine and 3nM paclitaxel has abolished the formation of primary mammospheres in by ~99% in MCF7 (p= 0.001) and SUM159 (p= 0.0001) cell lines. Cells initially propagated in the presence of 3µM ellipticine, barely produced any secondary mammospheres with very few cells passaged to third generation, compared to vehicle treated control. Representative pictures of primary mammospheres formed by MCF7 and SUM159 cell lines in the presence of varying concentrations of ellipticine and paclitaxel and their subsequent second and third generation mammospheres after drug withdrawal were shown in Figure 6.3. These findings suggest that ellipticine can reduce the proliferation and self renewal ability of ALDH1A1-positive BCSC population *in vitro* and can be used in combination with a cytotoxic drug like paclitaxel which helps in debulking of the tumor.

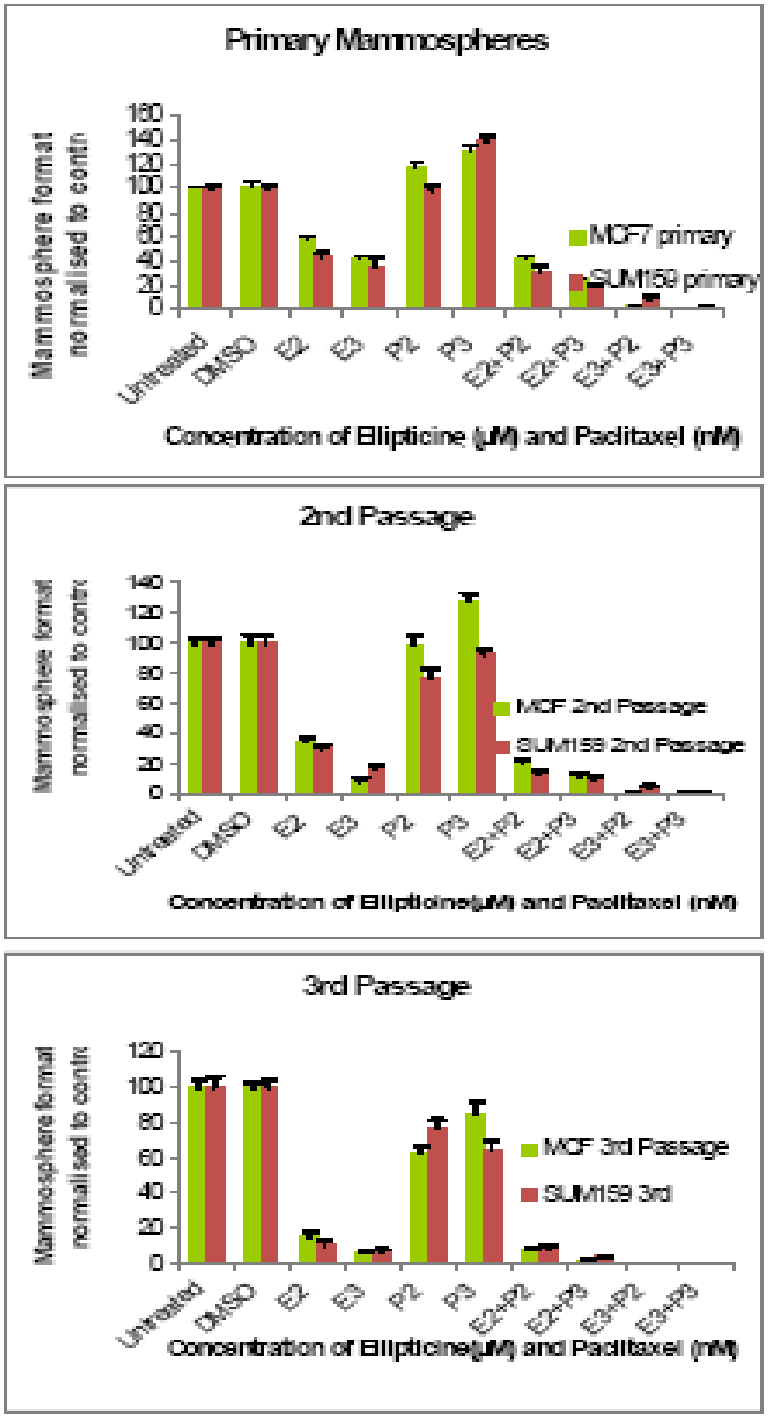


Figure 6.2. Inhibitory effect of ellipticine on mammosphere formation and their self renewal ability. Ellipticine abated the mammosphere enriching effect of paclitaxel and reduced the number of primary mammospheres. In the absence of drugs, the 2nd and 3rd passages that were derived from ellipticine and paclitaxel treated primary mammospheres yielded smaller number of spheres in comparison with control. Data are presented as mean ±SD (n=3)

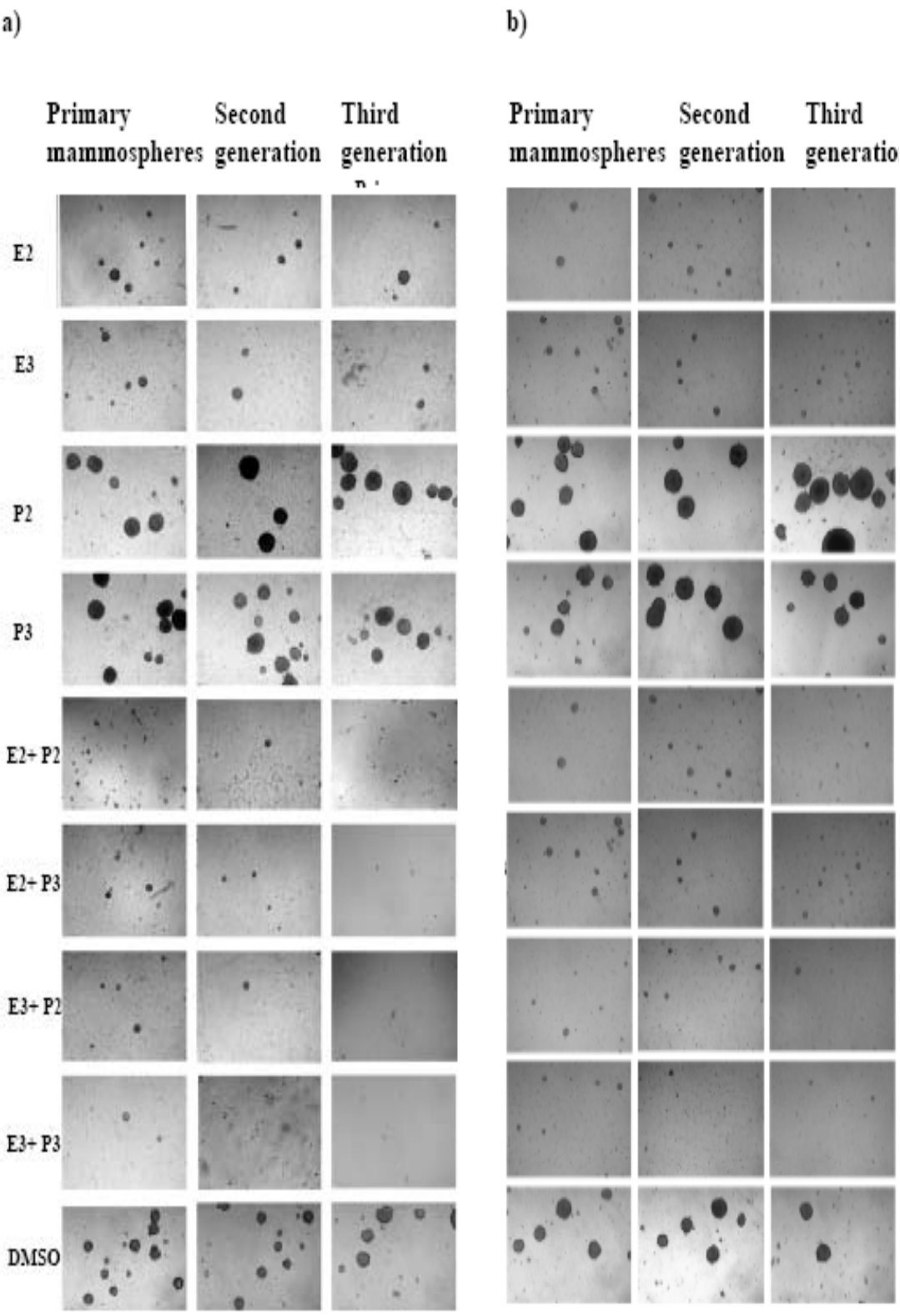


Figure 6.3. Representative pictures of primary mammospheres formed in the presence of varying concentrations of ellipticine and paclitaxel and their subsequent 2nd and 3rd generation mammospheres after drug withdrawal by (a) MCF7 and (b) SUM159 cell lines.

6.3.2. *In silico* studies

Cumulatively, our experimental evidence leads us to propose that ellipticine and/or its metabolically active derivatives might directly interact with ALDH1A1 enzyme and affect its biological activity which is indispensable in the maintenance of the stem cell pool. To further understand the molecular interactions and inhibitory mechanism of ALDH1A1 by ellipticine, we have performed molecular docking of ellipticine and its metabolic derivatives with the Homo sapien model of the enzyme preceded by homology modeling and molecular dynamics study of ALDH1A1.

6.3.2.1. Sequence analysis and homology modeling of Human of ALDH1A1

Blastp analysis revealed that primary structure of ALDH1A1 enjoyed 92.5% similarity with that of sheep liver class 1 ALDH (PDB ID 1BXS) and hence the later was chosen as a template for modeling of human ALDH1A1. For homology modeling, the multiple sequence alignments were followed by pairwise sequence alignment using ClustalW 2.0 between ALDH1A1 sequence and the template 1BXS. A total of 100 models of human ALDH1A1 were generated using Modellar9v11 and were superposed (Figure.4) on template crystal structure (1BXS) and the one with least RMSD on superimposition, with respect to trace Cα atoms of the crystal structure of template was chosen for further refinement and validation. The phylogenetic relationships between generated model of human ALDH1A1and its sequence related templates is given in figure 6.4.

6.3.2.2. Structural validation

The Procheck analysis of the energy minimized model of human ALDH1A1 showed that 100% of the residues were found in allowed regions of the Ramachandran plot. Moreover among the total amino acid residues of ALDH1A1 and 1BXS, 92.5% and 89.3% residues were positioned in the most favored regions of the Ramachandran plot (Table 6.1).

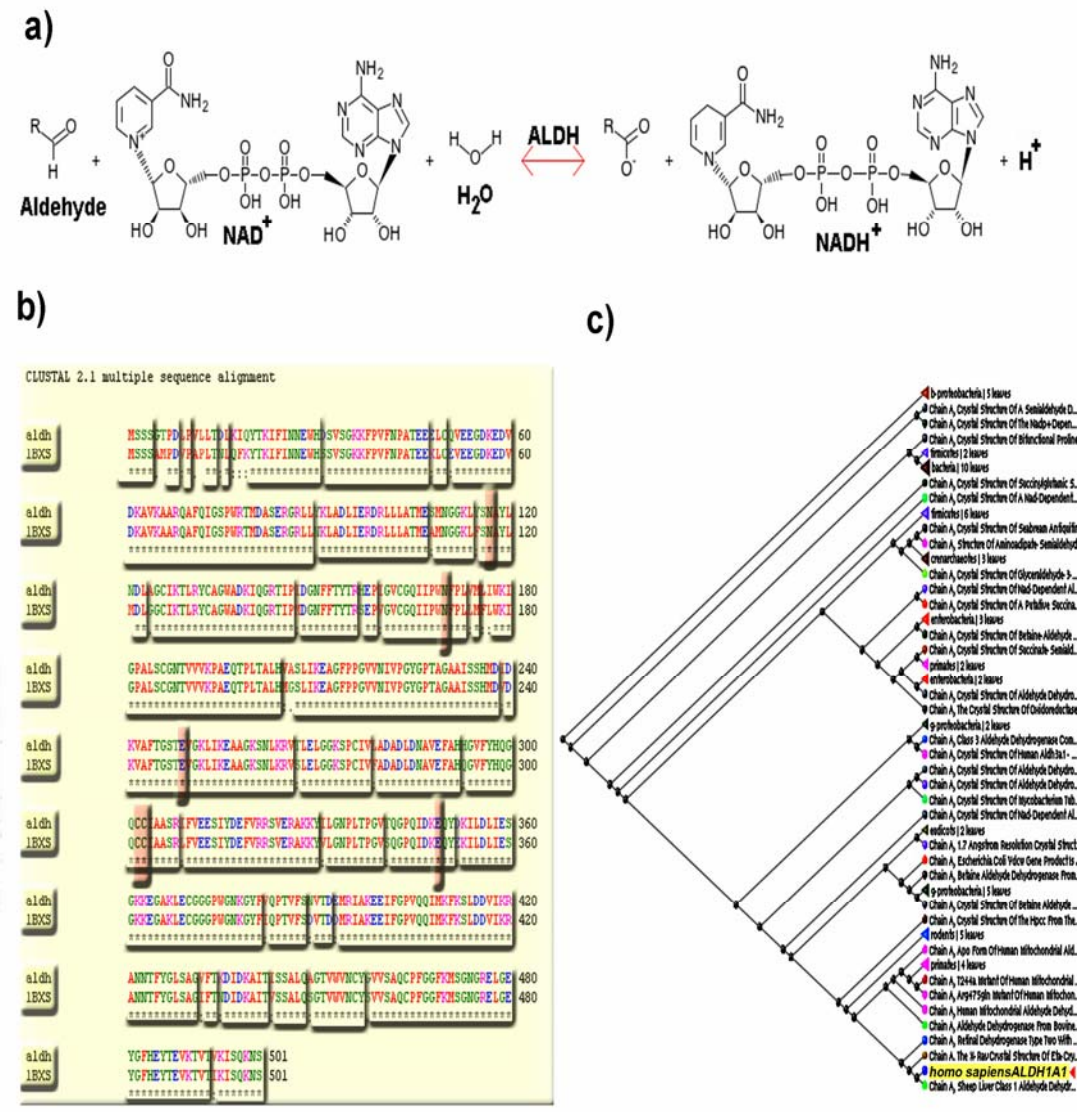


Figure 6.4. (a) Schematic representation of reaction catalyzed by ALDH. (b) Multiple sequence alignment of ALDH1A1 with its template 1BXS, conserved amino acids are indicated with asterisk, catalytic residues highlighted with light pink colour boxes. (c) Phylogenetic relationships between the generated model of Homo sapiens ALDH1A1 and its sequence related templates.

The total quality G-factor for human ALDH1A1 and for template 1BXS are 0.120 and 0.33 respectively, which indicates a good quality model (acceptable values of the G -factor in procheck are between 0 and −0.5, with the best models displaying values close to zero). Detailed secondary structural investigation of the predicted human ALDH1A1 model with Pdbsum , a secondary structure prediction server reveals 97 (19.4%) residues were in strands, 194 (38.7%) residues were in α-helices and, 4 (0.8%) residues were in 3–10 helix and 206 (41.1%) residues were in other conformations. (Figure. 6.5a). The tertiary structure of human ALDH1A1 (Figure 6.5b) shows close resemblance to crystallized 1BXS, with a back bone RMS value of 0.52 Å between ALDH1A1 and template-1BXS. The low overall RMS values for backbone superposition reflect the high structural conservation of this complex through evolution, making it a good system for homology modeling. The developed human ALDH1A1 protein model superimposed with template 1BXS is given in Figure. 5d. Also, the developed human ALDH1A1 protein model was submitted to protein model data base (PMDB) and a PMDB number (PM0078411) has been obtained. It show <3% stereochemical failures. Analysis of the energy minimized human ALDH1A1 model with WHAT-IF web interface reveals that RMS Z -Scores for bond angles and bond lengths are all close to 1 and also within the limits of templates (Table 6.1). Evaluation of the energy minimized model of human ALDH1A1 with ProSA- web reveals that the Z -score value (−10.05) is in the range of native conformations of the crystal structures. ProSA-web analysis had showed that overall the residue energies of the human ALDH1A1 model were largely negative except for some peaks in the middle region. The final structure has been further evaluated for overall quality by Verify 3D. The compatibility scores for all the residues were found to be above zero. ERRAT calculates the “overall quality factor” for non-bonded atomic interactions, and higher scores mean higher quality. The normally accepted range is >50 for a high quality model. In the current study, the ERRAT scores for the ALDH1A1 model and the template 1BXS are 84.239 and 98.039 respectively are well within the range of a high quality model (Table 6.1). Thus, the above analysis suggests that the backbone conformation and non-bonded interactions of human ALDH1A1 homology model is all reasonable within a normal range. WHAT-IF is used to check the normality of the local environment of amino acids. For the WHAT-IF

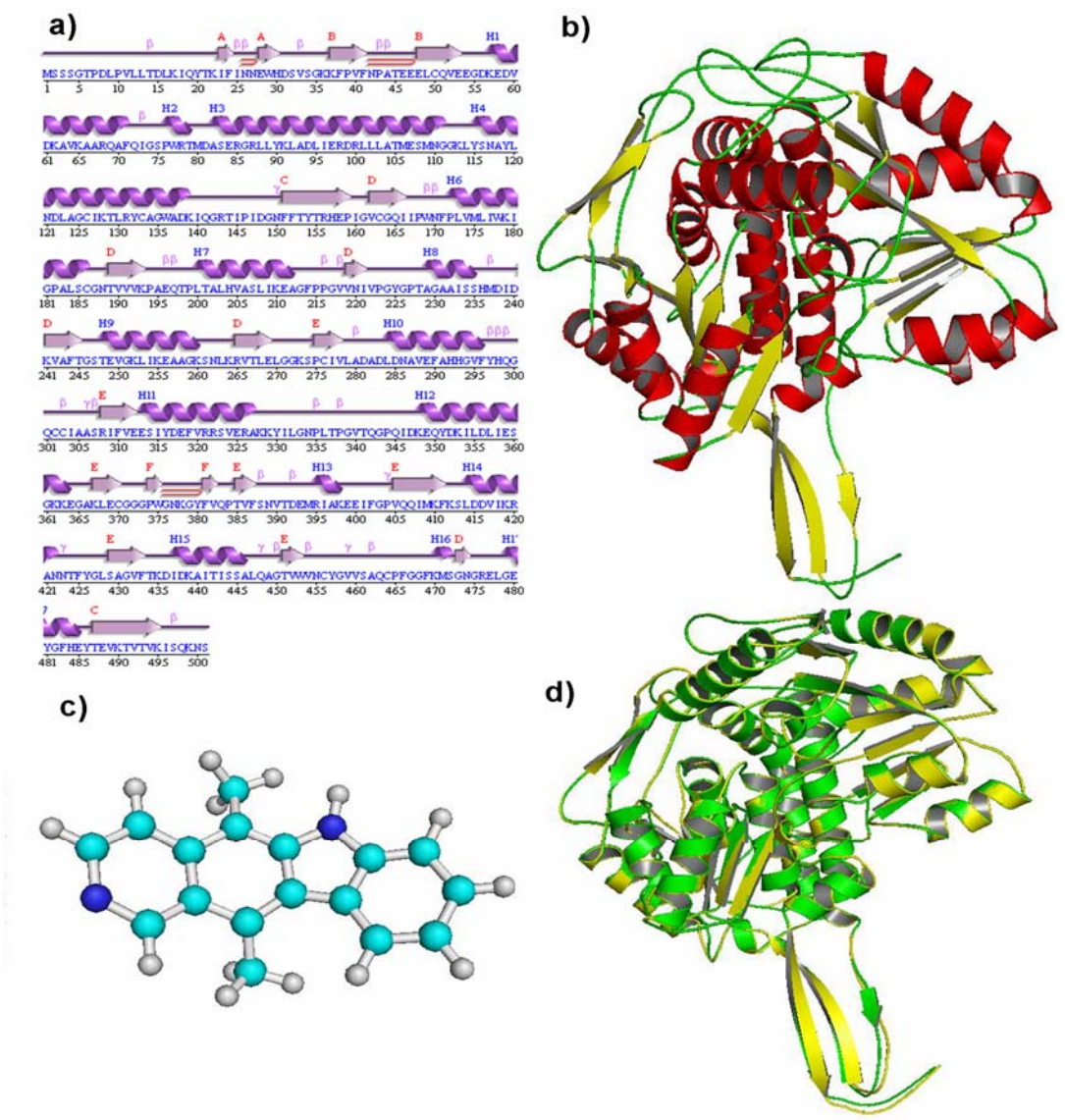


Figure 6.5. (a) Secondary structure of developed model of Homo sapiens ALDH1A1. (b) Developed three dimensional structure of Homo sapiens ALDH1A1. (Sheets are highlighted in yellow, helices in red and loops in green color). (c) 3D structure of ellipticine. (d) Developed 3D structure of Homo sapiens ALDH1A1 (Green) superposed with template 1BXS yellow). The superposition of the average structure of the human ALDH1A1 with the initial model did not show major structure conformational changes.

M1

PROCHECK								ERRAT
Ramachandran Plot Quality (%)					Goodness Factor			Score
	Most favored	Additional Allowed	Generously allowed	Dis-allowed	Dihedral	Covalent	Overall	
ALDH1 A1	92.5	6.6	0.9	0.0	0.01	0.27	0.12	86.094
1BXS	89.3	10.3	0.5	0.0	0.21	0.49	0.33	96.000

Table 6.1: PROCHECK and ERRAT

Structure	Avarage package quality[a]	Rotamer Normality[b] $\chi_1\chi_2$	Backbone Conformation	Bond length[c]	Angle[d]
ALDH1 A1	-0.453	-0.364	-0.809	0.910	1.212
1BXS	-0.055	-2.248	-0.282	0.469	0.705

[a]The average quality of 200 highly refined X-ray structures was -0.5 ± 0.4

[b]The behaviour of the these distribution is much that a Z-score below -2 (2 standard deviations way from the average) is poor, and a Z-score of less than -3 is of concern; positive is better than average.

[c]RMSD Z-score should be close to 1.0

[d]RMSD Z-score, more common values are around 1.55.

Table 6.2. WHATIF stereochemical quality evaluation

evaluation, the quality of the distribution of atom types is determined around amino fragments. For a reliable structure, the WHAT-IF packing scores should be above − 5.0. In this case, none of the scores for each residue in the homology model is lower than − 5.0 (Table 6.2). Therefore, the WHAT-IF evaluation also shows that the homology model structure is very reasonable.

6.3.2.3. Molecular dynamics analysis

To obtain a more reasonable structure, we performed nanosecond timescale molecular dynamics simulations for the structure of ALDH1A1. The protein backbone RMSD from simulations showed that the structure was equilibrated after 3000 ps of simulation. Thus, we selected the last 3 ns simulation results to obtain an average structure using the g_rmsf program of Gromacs, and plots of potential energy fluctuation. The root mean square fluctuation (RMSF) of the protein was measured during the last 10 ns of MD simulation, as the structure during the several nanoseconds was in an equilibrium state. ALDH1 A1 active site residues Asn117, 121 and Gln-350 are in less flexible part of the protein with minimum fluctuation in time. The RMSF of Glu-249 and Cys-302 are more flexible and are closely associated with the grid of the active site. Asn117, 121, Glu-249, Cys-302, and Gln-350 were found to be present in the most rigid part of the protein and thus these amino acids might play a key role in substrate specificity.

6.3.2.4. Docking studies of ellipticine and its derivatives with human ALDH1A1

Docking studies were performed to gain insight into the binding conformation of ellipticine (Ellipticine, 5, 11-Dimethyl-6H-pyrido [4, 3-b] carbazole) derived from NCBI Pubchem compound ellipticine (CID 3213) (Figure. 6.5c) and its derivatives, 7-hydroxyellipticine, 9-hydroxyellipticine, 12-hydroxyellipticine, N_2-Oxide ellipticine, 13-hydroxyellipticine with ALDH1A1. AutoDock run not only predicts a binding conformation but also produces a value for free energy of binding When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells (data not shown). Thus, we hypothesize that lack of mammosphere formation could not be attributed

to the absence of live cells beyond the sixth/seventh passage, but due to differentiation of cancer stem cells.

FEB) in kcal/mol and an estimated inhibitory concentration (Ki) in mM. All docking calculations were carried out using Auto Dock 4.0/ADT and the dlg files generated were analyzed for their binding conformations. Analysis was based on free energy of binding, lowest docked energy, and calculated RMSD values (Table 6.3). The developed docking conformations of ALDH1A1 with ellipticine and its metabolic derivatives: 7-hydroxyellipticine, 9-hydroxyellipticine, 12-hydroxyellipticine, N_2-Oxide ellipticine, 13-hydroxyellipticine showed negative binding energies; Among these the EDC-3 (12-hydroxyellipticine) and EDC-5 (13-hydroxyellipticine) shows best values of binding energy −9.02 kcal/mol, -9.59 kcal/mol and docked energy of -9.75 kcal/mol -9.35 kcal/mol respectively; their RMSD and inhibition constants were estimated to be 0.224A^o and 0.389 A^o respectively.

	Lead Molecule	Run	RMSD from reference structure (A^0)	Estimated Free Energy of Binding (kcal/mol)	Docked energy (kcal/mol)	Estimated Inhibition Constant, Ki uM (micromolar) [Temp= 298.15K]
1.	Ellipticine (Parental Compound)	20	0.604	-8.73	-7.23	4.88 uM
2.	EDC-1(7-hydroxyellipticine)	32	0.952	-7.37	-7.59	5.98 uM
3.	EDC-2 (9-hydroxyellipticine)	30	0.915	-7.40	-7.86	5.50 uM
4.	**EDC-3 (12-hydroxyellipticine)**	13	**0.224**	**-9.02**	**-9.75**	**2.10 uM**
5.	EDC-4 (N_2 Oxide of ellipticine)	09	0.970	-6.79	-6.57	7.57 uM
6.	**EDC-5 (13-hydroxyellipticine)**	46	**0.389**	**-9.59**	**-9.35**	**3.85 uM**

Table 6.3: Docking results of ellipticine and its derivative molecules docked on to ALDH1A1 model

S. No	Ellipticine Parental Compound	7-hydroxy ellipticine	9-hydroxy ellipticine	12-hydroxy ellipticine	N_2 Oxide of ellipticine	13-hydroxy ellipticine
Catalytic Residues	Asn-117	Asn-117	Asn-117	**Met-102**	Asn-117	Asn-117
	Asn-121	Asn-121	Asn-121	Asn-117	Asn-121	Asn-121
	Glu-249	**Asn-170**	**Asp-122**	Asn-121	**Thr-245**	**Pro-168**
	Cys-302	**Val-174**	Glu-249	**Asn-170**	Glu-249	**Asn-170**
	Cys-303	Glu-249	Cys-302	Glu-249	Cys-302	**Thr-245**
	Gln-350	Cys-302	Cys-303	Cys-302	Cys-303	Glu-249
		Cys-303	Gln-350	Cys-303	**Ile-304**	Cys-302
		Gln-350	**Try-453**	Gln-350	Gln-350	Cys-303
				Gly-458		Gln-350

Table 6.4. Interactions of catalytic residues with parental compound ellipticine and its derivatives. The amino acids Asn-117, Asn-121, Glu-249, Cys-302 & 303 and Gln-350 interact with the metabolic derivatives of ellipticine were highlighted with bold and underlined.

These features qualify EDC-3 & 5 as potential leads with most drug like properties (Table 6.3). Docking of human ALDH1A1 protein with ellipticine and its derivatives further revealed that the amino acids Asn-117 & 121, Glu-249, Cys-302 and Gln-350, present in the active site of the protein played vital role in binding to the drug compound best values of binding energy −9.02 kcal/mol, -9.59 kcal/mol and docked energy of -9.75 kcal/mol -9.35 kcal/mol respectively; their RMSD and inhibition constants were estimated to be 0.224A^{o} and 0.389 A^{o} respectively. These features qualify EDC-3 & 5 as potential leads with most drug like properties (Table 6.3). Docking of human ALDH1A1 protein with ellipticine and its derivatives further revealed that the amino acids Asn-117 & 121, Glu-249, Cys-302 and Gln-350, present in the active site of the protein played vital role in binding to the drug compound (Table 6.4) (Figure 6.6 & 6.7).

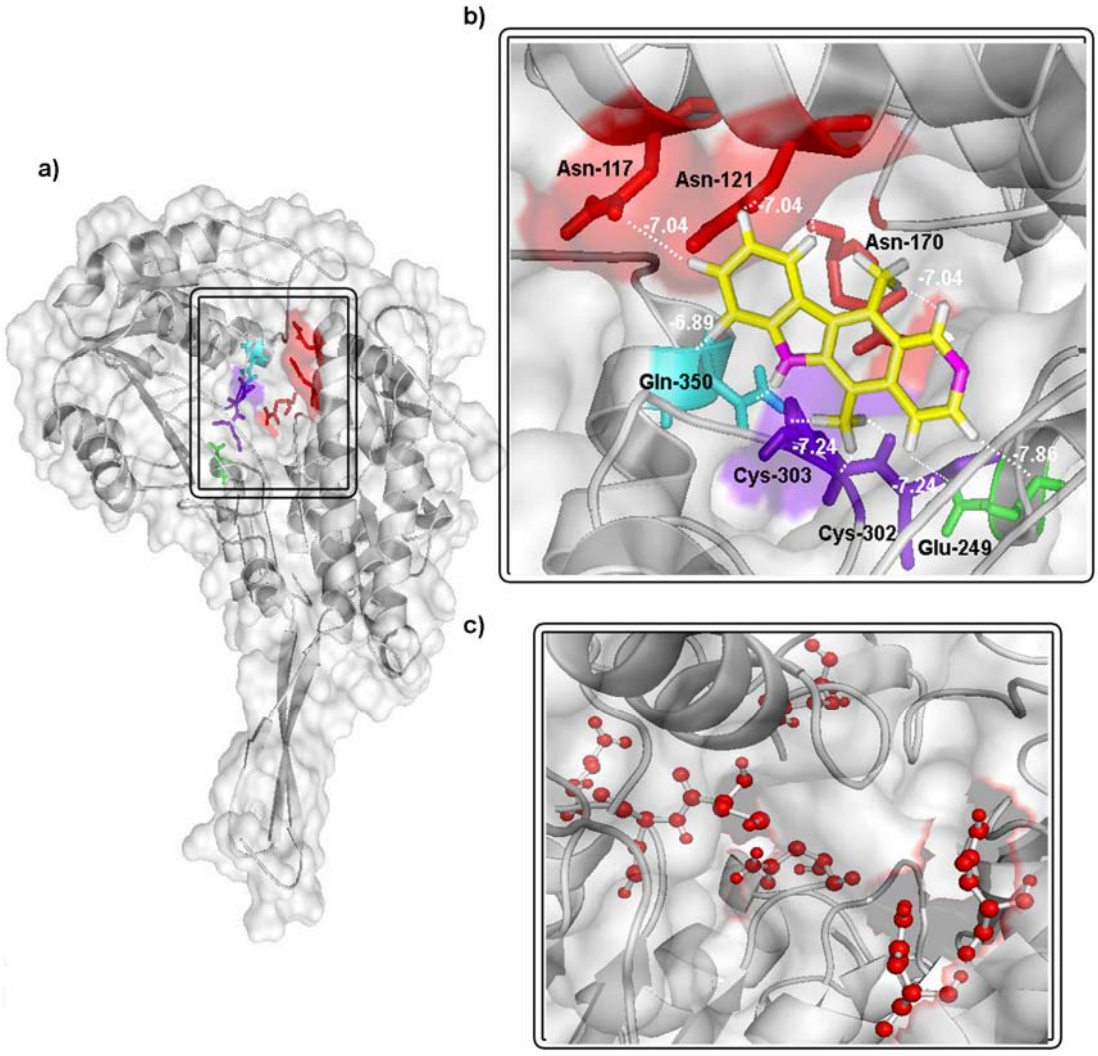


Figure 6.6. (a) Docking site of interaction of developed 3D structure of Homo sapiens ALDH1A1 protein with the drug compound ellipticine and its derivatives. (b) shows that the amino acids Asn-117 & 121, Glu-249, Cys-302 & 303 and Gln-350 played vital role to interact with parental compound Ellipticine, (c) catalytic residues of ALDH1A1 represented in ball and stick model (red color).

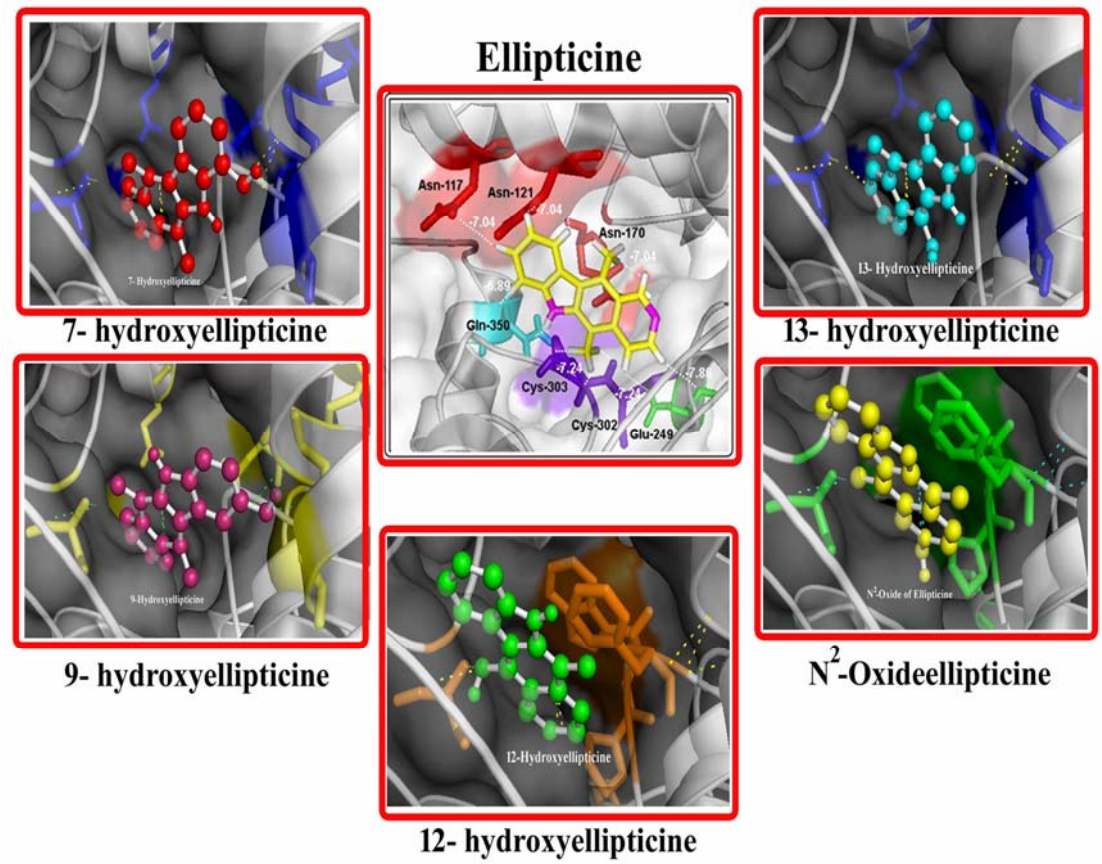


Figure 6.7. Docking interaction of developed 3D structure of Homo sapiens ALDH1A1 protein with the drug compound ellipticine and its derivatives. The amino acids Asn-117 & 121, Glu-249, Cys-302 & 303 and Gln-350, present in the active site of ALDH1A1 play a vital role in interacting with the parental compound Ellipticine. Other catalytic residues interacting with the metabolic derivatives of ellipticine were listed in table 4.

6.4. Discussion

Ellipticine exhibited significant anti-tumor and anti HIV properties in clinical trials. It showed activity against various cancer cell lines such as leukemias, lymphosarcomas, B16 melanoma, colon cancer, Lewis lung carcinoma, human non-small-cell-lung-cancer, hepatocellular carcinoma, glioblastoma, osteosarcoma, breast adenocarcinoma and neuroblastoma. Ellipticine has been reported to be an extremely promising anticancer drug due to its pleiotropic effect on various cancer cells [356-361]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects and their complete lack of hematologic toxicity [339]. Nevertheless, ellipticine is a potent mutagen. Most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa* and mammalian cells and induce prophage lambda in *Escherichia coli* [357].

Furthermore, ellipticine has been reported to play an important role in the restoration of mutant p53 activity [362]. Previously it was reported that p53 mutations could induce drug resistance atleast in part by interfering with normal apoptotic pathways in cells [363]. Godar *etal.*, [364] demonstrated that p53 represses the expression of CD44, a commonly used marker of breast cancer stem cells by binding to its promoter. This indicates that the loss of p53 function leads to increased expression of CD44, which promotes expansion of tumor initiating cells. Freed-Pastor and colleagues showed that depletion of the mutated form of p53 reverses the oncogenic potential of breast cancer cell lines by inducing a normal-like phenotype characterized by the formation of acini like structures [363]. But the association between aldehyde dehydrogenase activity and p53 status of the cell is not well understood. Interestingly, we have observed an almost equal magnitude of ALDH1A1-positive BCSC reduction in both MCF7 which possess a p53-wildtype and SUM159 cell line which carries a p53-mutant gene, when treated with ellipticine both alone and in combination with paclitaxel.

In breast tumors, the use of neoadjuvant regimens showed that conventional chemotherapy could lead to enrichment of CSCs in treated patients as well as in xenografted mice [253, 254]. Furthermore, primary mammospheres from chemotherapy treated patients showed similar mammosphere-initiating capacity after eight to ten passages, whereas cells from untreated patients vanished within two or three generations, suggesting again an increase in cells with self-renewal potential after chemotherapy [26, 253]. In MCF7, the progenitor cells isolated as mammospheres were found to be enriched with the CD44+/CD24-/low phenotype which were more resistant to radiotherapy than the bulk cells [365]. These findings strengthen the belief that CSCs resist and get enriched by conventional therapies and these are the actual targets to eliminate if treatment is to be curative. Dontu *et al* have previously demonstrated that BCSCs can be isolated from breast carcinomas based on their aldefluor-positive phenotype by Aldefluor assay that were both tumorigenic and metastatic in NOD/SCID mice [26]. They have used mammosphere culture to isolate and expand mammary stem/progenitor cells, based on their ability to grow as spheres under serum-free non-adherent conditions. Wayne Chou and group demonstrated that the down regulation of ALDH isozymes by siRNA, affected cell growth, cell motility, drug resistance and malignant transformation in lung cancer cells [366]. Thus, we utilized Aldefluor and mammosphere formation assay to evaluate the efficacy of ellipticine against BCSCs and demonstrated that ellipticine significantly suppressed the formation of mammospheres and also seems to abate the BCSC enriching effect of paclitaxel in breast cancer cell lines.

Findings of our mammosphere formation studies in the presence of varying concentrations of ellipticine and paclitaxel both alone and in combination, suggested that ellipticine can reduce the proliferation and self renewal ability of ALDH1A1 positive BCSCs in vitro and can be used in combination with a cytotoxic drug like paclitaxel which helps in debulking the tumor mass. An interesting observation is that ellipticine was able to inhibit the ALDH1A1-positive BCSC population *in vitro* at almost half the LC_{50} concentration of the drug that hardly effected the bulk population of cancer cells, implying that ellipticine is likely to preferentially target the ALDH1A1-positive BCSCs than the bulk cancer

cells. Another interesting finding is that there is a marked increase both in the number and size of the mammospheres formed by both MCF7 and SUM159 cells after exposure to paclitaxel relative to DMSO treated controls. This can be explained by the imperative role played by the "stem cell niche" in regulating stem cell behavior. Hence, we hypothesize that the cell-cell interactions, cell-extracellular matrix contacts, and response to growth factors may provide a unique niche to the breast cancer stem cells. Exposure of BCSCs to paclitaxel leads to the apoptosis of differentiated/bulk cancer cells leaving behind the breast cancer stem/progenitor cells thereby creating a unique "stem cell niche" which might encourage the cells to exhibit their maximum self renewal potential and a subsequent increase in sphere formation.

To determine the molecular level interactions (molecular simulations and protein-ligand docking) of human ALDH1A1 with ellipticine and its derivatives we extended our work *in vitro* to *in silico* studies. We developed high accuracy homology model of human ALDH1A1 using the crystal structure of NAD bound sheep liver class 1 aldehyde dehydrogenase (PDB ID: 1BXS) as a template. The quality of the homology model depends on the level of sequence identity between the templates of known 3D structures and the protein to be modeled. In this case, the level of sequence identity between the target and the template is 92.5%. In addition, the molecular dynamics simulation improved the general structure of the generated model. This model has been verified using several validation methods, including PROCHECK, ERRAT, WHAT-IF, PROSA2003 and VERIFY-3D. All evidences suggest that the geometric quality of the backbone conformation, the residue interaction, the residue contact and the energy profile of the structure are well within the limits established for reliable structures.

Our study forms the basis for the prediction of ALDH1A1-positive breast cancer stem cells' susceptibility to ellipticine and provides a strong rationale for the possibility of a new combinatorial chemotherapeutic regimen leading to improvements in the treatment of breast cancer. Further *in silico* data suggests that ellipticine reactive metabolites 13-hydroxyellipticine and 12-hydroxyellipticine could be good candidates for targeting breast tumors with high aldehyde dehydrogenase activity.

Chapter 7

Conclusions and Future Scope of work

Chapter 7: Conclusions and Future Scope of work

7.1. Conclusions

1. Two unique triple negative (for ER, PR and Her2/neu) breast cancer cell lines named NIPBC-1 and NIPBC-2 were established from primary tumors of two young breast cancer patients (39 and 38 yrs old respectively).

2. Both NIPBC-1 and NIPBC-2 were completely purified and passaged for 85 and 66 times respectively and characterised thoroughly.

3. Both NIPBC-1 and NIPBC-2 formed anchorage dependent colonies on soft agar confirming the transformed nature. Further, these cell lines showed positivity for invasion on matrigel confirming their malignant nature.

4. Karyotyping for both the cell lines shown aneuploidy with several translocations and rearrangements, particularly exhibiting 17q isochromosome.

5. C>G polymorphism at codon 72 has been found in p53 gene of NIPBC-2 cell line.

6. STR analysis has shown no matching with any other available cells lines representing their unique genotype.

7. Both the cell lines are typical of breast cancer cells with distinct ultrastructural, immunological, and genetic characteristics. These cell lines represent unique characteristics and may prove to be good experimental models for investigating breast cancer biology.

8. Both NIPBC-1 and NIPBC-2 can serve as *in vitro* models to study the early onset breast cancers in Indian women have been established.

9. We observed an increase in sphere forming efficiency from M1 to M2 (from 10.4% to 31.66%) in SUM159 cell line whereas it is from M1 to M3 in both MCF7 (5.8% to 25.8%) and HBL100 (4.9% to 23.8%) cell lines , but a decline thereafter among all the three cell lines

10. On analyzing the size of mammospheres through subsequent passages, we found that the size of the largest and smallest spheres in each passage remained almost consistent; however, the relative proportion of smaller spheres increased in number with passage.

11. We failed to detect mammosphere formation beyond the seventh passage in both MCF7 and SUM159 cell lines and beyond the sixth passage in the breast normal epithelial cell line after which, mostly single cells and few clumps/aggregates of cells were seen. When left in culture for more than 20– 25 days, mostly single cells with few cells containing large vacuoles were detected, but no new mammospheres formed even though trypan blue staining revealed 85% live cells.

12. Thus, we hypothesize that lack of mammosphere formation could not be attributed to the absence of live cells beyond the sixth/seventh passage.

13. Ellipticine phenomenally reduces aldehyde dehydrogenase 1 A1 (ALDH1 A1) positive Breast Cancer Stem Cells (BCSCs) as determined by Aldefluor assay.

14. Ellipticine and paclitaxel act synergistically on ALDH1 A1 positive BCSCs *in vitro*.

15. Ellipticine significantly suppresses mammosphere formation of ALDH1 A1 positive BCSCs in a dose dependent manner.

16. In the presence of varying concentrations of ellipticine and paclitaxel both alone and in combination, proliferation and self-renewal ability of ALDH1A1-positive BCSCs is reduced *in vitro*.

17. Ellipticine abates the BCSC-enriching effect of paclitaxel and can be used in combination with a cytotoxic drug like paclitaxel which helps in debulking the tumor mass.

18. 13-hydroxyellipticine and 12-hydroxyellipticine could be good candidates for targeting breast tumors with high aldehyde dehydrogenase activity.

7.2. Future Scope of work

Difference in clinical behavior and molecular profile of early onset breast cancer suggest the need for understanding the risk factors and molecular mechanisms involved in development of breast cancer in young women. Although a number of normal and breast cancer cell lines established from western patients are available, there is a paucity of cell lines established from Indian breast cancer patients. The two cell lines NIPBC-1 and NIPBC-2, which we have established in the present study are unique with respect to the ethnicity also these are the first breast cancer cell lines to be established from Indian continent representing early onset breast cancers. Hence may serve as *in vitro* models to study the early onset breast cancers in Indian women. In the present study we have demonstrated for the first time, the efficacy of ellipticine against breast cancer stem cells. Unraveling the effects of ellipticine on the molecular mechanisms and pathways associated with ALDH activity will help to further understand CSC biology and oncogenesis. Since cancer is a complex disease involving multiple molecular pathogenic pathways which are closely associated with each other, blocking or downregulation of any one pathway might compel the cell to take an alternative route. Hence, the present study warrants further mechanistic studies and application of these findings in breast cancer therapeutics.

Chapter 8

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Appendix

Preparation of Reagents

Acetic/Methanol: Add 1 part glacial acetic acid to 3 parts methanol. Make up this reagent fresh each time it is used and keep it on ice.

Calcium Chloride (0.1 M): 1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH2O and sterilized by autoclaving.

DEPC water: 0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37^0C it was autoclaved.

DNA loading dye (6X): 0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved ddH2O.

Ethidium Bromide (10 mg/ml): 10mg of ethidium bromide was dissolved in 1ml ddH_2O, stored in opaque bottle.

Methylcellulose (1.8%): Weigh out 7.2g of methocel and add it to a 500ml bottle containing a large magnetic stirrer bar. Sterilize by autoclaving with the cap loose for penetration of steam. Add 400ml of sterile MilliQ H_2O heated to 90^0C to wet the methocel. Stir at 4^0C overnight to dissolve. (The methocel will form a solid gel if the magnet does not keep stirring. The resulting solution is now methocel 2X, and for use, it should be diluted with an equal volume of 2X medium of choice. Use a syringe (without a needle) for dispensing.

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT Sigma). 50mg/ml in PBS. Sterilize by filtration.

Phosphate Buffer Saline (PBS) (1 litre) pH=7.4

8gm of Sodium Chloride (NaCl), 2gm of Potassium Chloride (KCl), 1.44gm of Disodium hydrogen phosphate (Na2HPO4) and 0.2gm of Potassium dihydrogen orthophosphate (KH2PO4), were dissolved in 800ml of ddH2O. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

Paraformaldehyde (PF) pH=7.4

4% Stock solution (100 ml): 4 gms of paraformaldehyde + 50 ml of distilled water + 10 ml of 10x PBS + few drops of 2M NaOH was heated on magnetic stirrer in a fume hood. When the solution became clear it was removed from the heat and after cooling pH was adjusted to 7.2. Finally volume was adjusted with distilled water to 100 ml.

1% Working solution (10 ml): 2.5 ml of Stock PF (4%) + 7.5 ml of 1x PBS.

List of Publications

List of Publications

1. **Santhi Latha Pandrangi**, Appalaraju BS, Sinha NK, Kumar M, Dada R, Lakhanpal M, Soni A, Malvia S, Simon S, Chintamani C, Mohil RS, Bhatnagar D, Saxena S. ***Establishment and characterization of two primary breast cancer cell lines from young Indian breast cancer patients: mutation analysis.*** Cancer Cell Int. 2014 Feb 5;14 (1):14.

2. **Santhi Latha Pandrangi**, Chikati R, Chauhan PS, Kumar CS, Banarji A, Saxena S. ***Effects of ellipticine on ALDH1A1-expressing breast cancer stem cells-an in vitro and in silico study.*** Tumour Biol. 2014 Jan;35(1):723-37. doi: 10.1007/s13277-013-1099-y. Epub 2013 Aug 28.

3. Gundampati Ravi Kumar, Rajasekhar Chikati, **Santhi Latha Pandrangi**, Manoj Kandapal, Kirti Sonkar, Neeraj Gupta1, Chaitanya Mulakayala , Medicherla V. Jagannadham, Chitta Suresh Kumar, Sunita Saxena, Mira Debnath (Das). ***Molecular docking and dynamics simulations of A.niger RNase from Aspergillus niger ATCC26550: for potential prevention of human cancer***. Journal of Molecular Modelling, 19 (2013) 613-621

4. Lakhanpal M, Yadav DS, Thoudam RD, Khangembam JS, **Santhi Latha Pandrangi,** Chauhan PS, Singh LC, Verma Y, Sharma J, Saxena S, Kapur S. ***Association of Interleukin-1β -511 C/T Polymorphism with Tobacco Associated Cancer in Northeast India: A Study on Oral and Gastric Cancer.*** II: S2210-7762 (14)00013-1, doi:10.1016/j.cancergen.2014.01.002

Participations

- Attended the National workshop on "**Chemoinformatics and Computational Drug Design**", sponsored by Department of Biotechnology, Govt. of India, organized by Biotech Park, Lucknow from February 27th – 29th, 2012.

- Participated in the workshop and Hands-on Training Course on "**Molecular Techniques in Cell Signalling and Biomedical Nanotechnology"**, organized by Department of Biochemistry, Banaras Hindu University, Varanasi, India from January 27th -31st, 2010.

- Attended the National workshop on **"Molecular Cytogenetics: Cancer Cytogenetics (solid tissue) by FISH"** organized by Department of Reproductive Biology AIIMS at New Delhi, India from August 24th to 29th, 2009.

- Attended National symposium on "Cancer Stem Cells" held at Panjab University, Chandigarh from 27th – 28th July, 2008.

- Attended National symposium on "Clinical Genomics" a satellite meeting of HGM held at India Habitat centre, New Delhi on 25th September 2008.

- Ravi Kumar Gundampati, **Santhi Latha Pandrangi**, Sunita Saxena, Mira Deb Nath. Poster presented on **"Anticancer activity of *A.niger* RNase (T2-family) from *Aspergillus niger* ATCC26550"**, World Congress on Biotechnology, Hyderabad, India from March 21st -23rd 2011.

- **Santhi Latha Pandrangi**, Sunita Saxena. Poster presented on **"Self renewal potential of Breast Cancer Stem Cell (BCSC) mammospheres in long term cultures"**. 3rd International conference on stem cells and

cancer (ICSCC-2012); proliferation, differentiation and apoptosis" held at Ram Manohar Lohia hospital, New Delhi from 27th to 30th October, 2012.

- **Santhi Latha Pandrangi**, Rajasekhar Chikati, Chitta Suresh Kumar, Sunita Saxena. Poster presented on **"Targetting of Homo sapiens Aldehyde dehydrogenase 1 class A1 enzyme with ellipticine as a novel strategy for breast cancer therapeutics- an *in silico* study"**. 32nd Annual Convention of Indian Association for Cancer Research and International Symposium: Infection & Cancer at University of Delhi from 13th -16th February, 2013.

Brief Biography of the Candidate

Brief Biography of the Candidate

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Educational Qualification

Educational Qualification	**Subjects studied**	**Year**	**University**	**Percentage**
B.Sc.	Biochemistry, Botany, Chemistry	1997	Andhra University, Visakhapatnam.	**76%**
M.Sc	Biochemistry	1999	Andhra University, Visakhapatnam.	**72%**
M.Phil	Biochemistry	2005	Bharatidasan University	**83%**
Ph.D	Cancer Biology	2007- Till date	Birla Institute of Technology and Science, Pilani.	

Title of Ph.D Thesis

"**Study on effects of anticancer drugs on breast cancer stem cells (BCSCs)",** under the guidance of **Dr. Sunita Saxena**, Scientist-G, Director, **National Institute of Pathology (ICMR)**, Safdarjung hospital campus, New Delhi. Registered for Ph.D at Birla Institute of Technology and Science (BITS-Pilani), Rajasthan, India

Research *Experience*

Company/Institute	Designation	Period
Hyderabad Central University	Junior Research Fellow	Feb 2000- Sept 2001
National Institute of Pathology	Senior Research Fellow	May 2007-Current

Research projects worked on

- Development of Low Cost Biosensors with Long Shelf Life for Medical Use, a BARC sponsored Project
- Biochemical and structural characterization of proteases from Soil bacteria
- Establishment of Breast cancer cell lines from primary breast tumours
- Study on effects of anticancer drugs on breast cancer stem cells (BCSCs)

Academic awards/ Achievements

- Awarded gold medal in B.Sc, for topping in Biochemistry.
- Baba Atomic Research Centre (BARC) - Junior Research Fellow, 2000.
- Pursuing Doctoral Program from May 2007 onwards at NIP as ICMR-SRF.
- Awarded ***independent senior research fellowship from Indian Council of Medical Research*** in August 2011 to work at National Institute of Pathology, New Delhi.
- Awarded ***travel grant by Council of scientific and Industrial research (CSIR)*** (Ref. No: TG/8011/13-HRD, dated 19 July 2013)
- Awarded ***financial assistance of Rs. 1,26,093, by Department of Biotechnology*** (DBT/CTEP/02/201300768) to attend "International Conference of Epidemiology and Evolutionary Genetics", Florida, USA.
- Awarded ***ICMR International travel grant for Non ICMR scientists*** (No.3/2/TG-42/HRD-2013 (9))

Teaching Experience :

- Worked as a regular Lecturer in M.S.Ramaiah college of Arts, Science and Commerce, Bangalore from Sept 2002 to August 2006.
- Taught B.Sc and M.Sc Biochemistry classes various papers viz., Biomolecules, Biochemical Techniques, Bioenergetics, metabolism, Cell Biology, Molecular Biology, Recombinant DNA Technology and Biotechnology and Immunology.
- Worked as both external and internal practical examiner in Biochemistry of the Bangalore University.
- Valued the Bangalore university theory Biochemistry papers.

Basic Details

Date of Birth	1st May. 1978
Languages	English, Hindi, Telugu, Kannada
Marital status	Married

References

Dr. Sunita Saxena, Director,
National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi, INDIA Mobile No-91-9868112120 Tel No-91-011-26198402-6 Fax No-91-011 26198401, 2616135 Email: sunita_saxena@yahoo.com

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Brief Biography of the Supervisor

Brief Biography of the Supervisor

Name	:	**Dr. (Mrs.) Sunita Saxena**
Designation	:	**Director**
Address	:	National Institute of Pathology-ICMR Safdarjang Hospital Campus, Post Box No.4909, New Delhi – 110029
Academic Qualifications	:	**M.B.B.S., D.C.P., M.D.(Path)**

Degree	Institute	Year	Remarks
M.B.B.S.	M.L.N.Medical College, Allahabad187	1974	
D.C.P. (Clinical Pathology	L.L.R.M.Medical College Meerut	1978	**Received Gold Medal & Merit Certificate**
M.D. (Pathology)	-do-	1981	

Details of Employment

Post	Duration	Institute
Research Officer	April 1981 to Dec.1985	Institute of Pathology, New Delhi.
Sr. Research Officer	Jan.1986 to Jan.1991	-do
Asst. Director	9th Jan.1991-2nd May, 1994	-do-
Deputy Director	2nd May, 1994 to 2nd May,'99	-do-
Deputy Director (Sr.Gr)	3rdMay, 99 to 8thApril, 2002	
Deputy Director (Sr.Gr) & Officer In charge	9thApril, 2002to 13th Dec., 2004	-do-
Director	14th Dec., 2004 till date	-do-

Areas of Specialization : **Molecular Oncology, Oncopathology**

Areas of Interest **Breast Tumors,**

Tobacco Associated cancers

Genito urinary cancers

Membership of National and International bodies

International: - Life Member International Union against Cancer (U.I.C.C.) **National:** Life Member: Indian Association of Cancer Research (IACR).

Life Member: Indian Association of Pathologists and Microbiologists (IAPM).

Life Member: Association for the promotion of DNA fingerprinting and other DNA technologies (ADNAT)

Life Member: Proteomic Society of India

Life member: Human Genomic Organization (HUGO)

Trainings Received:-

1. Trained for **'Culture of fastidious cells and modern techniques of cell manipulation'** at *National Facility for animal Tissue and cell culture, Pune* in Dec., 1991.
2. Trained in **'Genetic mutation detection techniques for BRCA 1 and BRCA2 genes in genomic DNA of Breast cancer patients'** at *Unit Genetic Epidemiology* at *International Agency for Research on Cancer, Lyon, France* in 1998.
3. Attended **Hands-on Training Course on Proteomics and DNA Micro arrays** held from 25th February to 10th March 2003 at CCMB, Hyderabad.

Fellowships:-

1. Awarded **WHO fellowship** to work on **Renal Pathology** at **St. Vincent's Hospital, Melbourne, Australia**, 1987.
2. Awarded **Yamigawa Yoshida Fellowship of U.I.C.C.** to work on *"Mutation analysis of BRCA1 and BRCA2 genes in Breast Cancer Patients***"** at unit of Genetic Epidemiology **International Agency for Research on Cancer, Lyon, France** in 1998.

Awards and Honors Received:-

1. Awarded **Gold Medal** and **Merit Certificate** for securing highest marks in **Diploma in Clinical Pathology** (D.C.P.).
2. **K. C. Basu Mullick award** for best research work by **Indian Association of Pathologists and Microbiologists for year 2008.**

3. Received '***NOVARTIS ORATION AWARD 2006***" of Indian Council of Medical Research for her work on Breast cancer on 18th Sept. 2009.

4. Elected ***Fellow of National Academy of Medical Sciences in 2010.***

5. Elected ***Fellow of Indian College of Pathologist in 2010***

Research Grants received:-

1. Awarded research grant to work on **"Genetics of Breast Cancer in Indian Women"** by *Indo-French Centre for Promotion of Advanced Research,* **New Delhi (1999-2002).**
2. Awarded Research grant from *Deptt. of Science and Technology* for proposal entitled **" A new approach to the management of superficial bladder cancer. Role of in vitro Cytotoxicity assessment and immunologic enhancement"** (2000-2003).
3. **"Comprehensive study of carcinoma esophagus at North-East India-Multidiscipline approach"**. Collaborative, Multicentric ICMR Task Force project (2004-2007).
4. **"Microsatellite instability in androgen receptor gene and mismatch repair system in Prostate cancer in Indian males"** – Department of Science Technology (2005-2008).
5. **"Role of tobacco use in causation of cancer in north-east India"** – Indian Council of Medical Research Task Force project (2005-2008).
6. **"Effects of pesticide exposure in causation of cancer in north-east India"** – Indian Council of Medical Research Task Force project (2005-2008).
7. **"Establishment of Cell lines from Primary Breast Cancer"** – Indian Council of Medical Research. Task force project (2007-2010).

8. **"Study on Gene Expression and Hypermethylation Profiles in Early Onset Breast Cancer"** Department of Biotechnology (2008-2011)
9. "**Characterization of host immune factors associated with progression of superficial TCC of bladder by microarray analysis"** Indian Council of Medical Research (2009-2012)
10. "**Immunogenetic profile of Nasopharyngeal Cancer in a high prevalence region of Northeast India"** Department of Biotechnology (2010-2013)
11. **"Comparative study of Genetic, Clinical and Epidemiological Factors of Breast Cancer in Rural and Urban Area of India"** Indian Council of Medical Research Task force project (2009-2012).
12. **"Epigenetic studies in esophageal cancer in high risk region of Northeast India"** Department of Biotechnology, Twining Program for NER (2011-2013)
13. "**Genome wide Analysis of Genetic alterations in patients with Esophageal Cancer from Northeast India using Single Nucleotide Polymorphism arrays"** Indian Council of Medical Research (2011-2013)
14. **"Study on miRNA signatures associated with Breast cancer stem like cells (CSC) and their role in drug response"** Indian Council of Medical Research (2012-2015)
15. **"Targeted sequencing of Breast cancer specific genes in early onset breast carcinoma."** Indian Council of Medical Research (2013-2015)

Academic Experience:-

a. Diplomat of National Board Examinations (N.B.E.) – Institute is accredited for running this program since 1993.
   - ***Core member, Supervisor & examiner***.
   - Guided 20 DNB dissertations as supervisor and co supervisor.
   - Nominated as Inspector, Examiner for theory & Practical examinations and paper setter.

b. Ph.D. - ***Supervisor/Mentor***

- ***Supervisor*** of student registered under GGSIPS University, Delhi, BITS, Pilani, Symbiosis, Pune and Jamia Hamdard University, Delhi
- External Examiner for Ph.D. candidates of AIIMS, PGI, Chandigadh, Agra University, BITS, Pilani.
- 4 students completed Ph.D and 9 are registered.

c. Member of project Review Committee of Divisions of ***Non Communicable Diseases of Indian Council of Medical Research.***

d. Member of project Review Committee of Divisions of ***Basic Medical Sciences of Indian Council of Medical Research.***

e. Member of ***Scientific Advisory committee of Institute Of Cytology and Preventive Oncology, Noida***

f. Reviewer of papers of ***Indian Journal of Medical Research, Human Mutation, Cancer Immunology Immunotherapy, World Journal of Surgical Oncology***, **BMC Cancer*, Cancer Detection and Prevention, British Journal of Urology International, Indian Journal of Medical Research, PLoS One***

g. Member of ***Scientific Advisory committee of National Jalma Institute for Leprosy and other Mycobacterial diseases, Agra.***

h. Member of ***Scientific Advisory committee of Regional Medical Research Centre, Dibrugadh***

i. Member of ***Scientific Advisory Group of Publication & Information Division Of Indian Council Of Medical Research***

j. ***Chairperson of Institutional Ethical Committee of Safdarjang Hospital, New Delhi.***

k. Member of ***Technical committee of Indian Council of Medical Research***

l. Member of **DBT sponsored DSMB on Curcumin Trial in Cancer Cervix**

m. **Senate member of BITS, Pilani.**

n. Nominated as the expert member of **Task force on Leprosy at ICMR.**

o. Nominated as the member of Scientific Advisory Group for creating a new centre for Environmental Health and Bhopal Gas Tragedy at Bhopal.

p. Nominated as the member of the Data Safety Monitoring Board (DSMB) on "BASANT Clinical Trial" of DBT, New Delhi.

q. Appointed as **Appraiser and Inspector** by National Board of Examination
for assessment of DNB students and institutes.

q. Dr. Sunita Saxena has been nominated as the expert member of " ICMR-ICAR Joint Task force on the Epidemiology of Human and Animal Brucellosis".

r. Dr. Sunita Saxena has been nominated as **nodal officer for getting ICMR university status.**

International Conferences attended.

1. Presented a paper on **"Pattern of lymphokines in minimal change Nephrotic syndrome"** in 5th *Asia Pacific Congress of Nephrology* held in New Delhi during 9-12th Dec., 1992.
2. Presented paper on **'Role of Proto-oncogene, Growth Factor Receptor and Steroid Hormones on Malignant Human Mammary Epithelial Cancer Cells in vitro and vivo"** in XVI, *International Cancer Congress (U.I.C.C.)* at New Delhi, 30th-5th Nov., 1994.
3. **'Stage A carcinoma of Prostate'** paper presented at *first conference of Nephrology, Urology and Transplantation Society of SAARC Countries* held at A.I.I.M.S. , New Delhi during 24th-26th March, 1995.
4. **'Mutation profile of BRCA 1 / 2 genes in Indian patients'** paper presented at *XV Asia Pacific Cancer Congress* held at Chennai during Dec. 12-15, 1999.
5. **'Mutation profile of BRCA 1 / 2 mutations in worldwide population. The MAGIC project'** paper presented in meeting of *American Association of Cancer Research AACR*, 2001, LA, USA.
6. Attended the 7th International Symposium on Molecular Basis of Predictive Oncology and Intervention Strategies' and presented a paper **BRCA1 and BRCA2 Genes in Indian Breast Cancer Patients** held at Nice, France from 7th 10th Feb. 2004.
7. Attended the **"UICC World Cancer Congress and Centre for Disease Control and Prevention (CDC)"** held during 8th to 13th July, 2006 at

Washington DC, U.S.A and presented paper **"Study of candidate genes associated with Breast Cancer Susceptibility in the Indian Women".**

8. Attended the NCRI Cancer Conference held at International Convention Centre in Birmingham, UK from 30th September - 3rd October 2007 and presented paper (oral and poster) entitled **"Gene expression profile of esophageal cancer in North East India by cDNA microarray analysis"**

9. Attended World Cancer Congress-2008 held in Shanghai, China during 12th-17th June 2008 and presented paper **"Differential gene expression in familial and tobacco associated esophageal cancers in north-east region of India".**

10. Attended HUGO's 13th Human Genome Meeting, September 27 - 30 2008, Hyderabad. and presented papers-

- **Prognostic value of TP53 Codon 72 polymorphism in oral cancer and stomach cancer in high risk region of India**
- **Differential gene expression profile of stomach and oral cancer in high risk region of India.**
- **Differential expression of MAPK and GPCR pathway in esophageal cancer of North-east region of India**
- **Significance of TP53 codon 72 polymorphism in breast and lung cancer showing different xenobiotic potential spectrum**

11. Attended **First Symposium on HPV Vaccination in the Asia Pacific and Middle East Region held at Seoul, Korea** during1st to 3rd June'09.

12. Visited **University of Minnesota, USA as member of expert team of Indian Scientists on Cancer** for collaborative research projects.

13. Presented papers in **Conference of the Organisation for Oncology and Translational Research (OOTR), 6th Annual Conference on 26 and 27 February, 2010 at Kyoto Japan**

- Genetic alterations in patients with esophageal cancer from high-risk region in India by SNP array. *Sujala Kapur, Indranil Chattopadhyay, Rupkumar hukan, Joydeep Purkayastha, Vikki Marshal, Amal Kataki, Jagdish Mohanta, David Bowtell, Sunita Saxena*
- Genome-wide analysis of genetic alterations in breast cancer patients from Northeast India using 10K SNP arrays. *Sunita Saxena, Mishi Kaushal, Indranil Chatterjee, A. Bhatnagar, Chintamani, D. Bhatnagar, Sujala Kapur*

14. Presented papers in **20th Asia Specific Cancer Conference, November 12-14, 2009,Japan.**

- GENOME-WIDE ANALYSIS OF GENETIC ALTERATIONS IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA BY SNP ARRAY. Sujala Kapur, Indranil Chattopadhyay, Rupkumar Phukan, Joydeep Purkayastha, Vikki Marshal, Amal Kataki, Jagdish Mohanta, David Bowtell, Sunita Saxena.

- GENOME-WIDE ANALYSIS OF DNA COPY NUMBER VARIATIONS IN INDIAN BREAST CANCER PATIENTS USING HIGH-DENSITY SNP ARRAYS. Sunita Saxena, Mishi Kaushal Wasson, Indranil Chatterjee, A Bhatnagar, Dr Chintamani, D Bhatnagar, Sujala Kapur.

15. Presented paper entitled "**Molecular Profile of Esophageal Cancer in High Risk Region of India"** in 10th World Congress of OESO held at Boston, USA during 28-31 Aug'2010. (Abstract J. Clin. Gastroenterology. Vol: 45 (2), 2011.
16. Presented paper entitled "**Genomic alterations in breast cancer patients from Northeast India using 10K SNP arrays"** in BMC group conference **Beyond the Genome: The true gene count, human evolution and disease genomics** at Harvard Medical School, Boston, USA during11th-13th Oct'2010
17. Presented paper entitled "**Betel Quid Chewing A Risk Factor For Breast Cancer: Study Of Genomic Alterations"** at 16th Human Genome Meeting 2012 held at Sydney, Australia during 11th-14th March, 2012

Publications: -

Research Articles:

1. Chandra M., **Saxena, S.**, Dave P.K., Kaza R. M. and Saxena, H.M.K.: **Proliferative myositis (a pseudosarcomatous lesion of skeletal muscle) occurring in an infant**. *Ind. J. Path. & Microbiol :* 26, 213, 1983.
2. Nagar P., **Saxena S.** (nee Sinha). Pratap V.K. and Mehrotra M.L.: **Malignant haemangiopericytoma of the uterus**. **A case report with brief review of literture**. *J. of Obst. Gynaec. Of India : 34,753, 1984.*
3. **Saxena S.**, Andal, A. and Saxena H.M.K.: **Ultrastructure study of minimal change nephrotic syndrome – a clinico-morphologic correlation**. *Ind. J. Med. Res.* 82: 171, 1985.
4. **Saxena S.,** Andal A, and Saxena H.M.K. **Stereomicroscopic examination of kidney tissue for rapid identification of glomerulus**. *Nephron:* 45: 249, 1987.
5. **Saxena S.,** Mehrotra M.L.: **Host tissue response in soft tissue sarcomas.** *Ind. J. Path. & Microbiol*. 30:97, 1987.
6. **Saxena S.,** Andal A, and Saxena H.M.K. **Idiopathic nephrotic syndrome of childhood: Ultrastructural immunohistologic and Clinicocomorphologic correlation.** *Ind. J. Path. & Microbiol.* 31 (3) 195, 1988.

7. Andal A, **Saxena S**, Chellani H.K. and Sharma S. **Pure Mesangioproliferative Glomerulonephritis. A Clinicomorphologic analysis and its possible role in morphological transition of minimal change lesion to Focal glomerulosclerosis**. *Nephron:* 51(3): 314, 1989.
8. **Saxena S,** Davies D.J., Krisner R.L.G. **Thin basement membrane in minimally abnormal glomeruli.** *J. Clin. Pathol*. 43: 32, 1990.
9. **Saxena S.**, Andal A, Saxena R.K., Sharma S, Chandra M, Saxena H.M.K. **Immune status of children suffering from Minimal change nephrotic syndrome**. *Ind. J. Path. & Microbiol.* 35(3) 171, 1992.
10. **Saxena. S.**, Davies D.J., **Glomerular alterations in Idiopathic haematuria– Ultrastructural and Morphometric analysis**. *Ind. J. Path. & Microbiol.* 35(4), 326-332, 1992.
11. **Saxena. S.**, Andal. A., Sharma. S, Saxena H.M.K., Chandra M. **Immunomodulation by measles vaccine in children with Minimal change nephrotic syndrome**. *Indian J. of Nephrology* 2, 141-146, 1992
12. Verma. A.K., Tandon, R., **Saxena. S.**, Pandy, J., Talib. V.H. **Aspiration Cytology of maxillary myxoma**. *Diagnostic Cytopathology* 9(2), 202-204, 1993.
13. **Saxena S**, Mital. A, Andal A,: **Pattern of interleukins in MCNS of childhood**. *Nephron* 65(1) 56-61, 1993
14. **Saxena S.**, Bhargawa R., Mohanty N.K., Talwar M: **Primary adenocarcinoma of the urinary bladder. A case report with review of literature** *Ind J Pathol and Microbiol.* 37(4), 453, 1994.
15. Saha T.K., Jolly B B., Mohanty N.K., **Saxena S.**, Dawson. L. **Multiple stones in Ectopic megaureter with Dysgenetic kidney – A case report**. *Ind. J. Nephrol*. 4(2). 61, 1994.
16. **Saxena S**. **Cytokine growth factors and childhood nephrotic syndrome**. *Jr. of Nephrol*. Vol. 8(6), 287, 1995.
17. Mohanty NK, Jolly BB, **Saxena S**, Dawson L. **Squamous cell carcinoma of peripheral urethrostomy**. *Urol. Int.* 1995, 55: 118-119.
18. Mohanty NK, Jolly BB, Talwar M, **Saxena S**, Dawson L. **Aspergillosis kidney. A case report**. *Indian Jr. of Nephrol*. 6(2), 56-58, 1996.
19. **Saxena S**, Jain A K, Pandey K K, Dewan A K. **Study on role of Steroid Hormone Receptors, Growth factor/receptors and Proto-oncogenes on behavior of Human Mammary Epithelial cancer cells in vitro.** *Pathobiology* 65(2), 75-82, 1997.
20. **Saxena S**, Mohanty N K, Talwar M, Jain A K. **Screening of Prostate Cancer in males with prostatism**. *Ind. J. of Path & Microbiol*. 40(4), 441-450, 1997.
21. Mohanty N K, Gulati P, **Saxena S**. **Role of interferon α-2b in the prevention of superficial carcinoma of bladder recurrence**. *Urol. Intern*.59: 194-196, 1997.
22. Mohanty N.K., Jha AK, **Saxena S**, Kumar S., Arora RP. **Ten years experience with Adujuvant Intravesical Immunotherapy in management of superficial transitional cell carcinoma of Urinary bladder – A review**. *Ind. J. of urology* 2001, 17, 127.

23. **Saxena S**, Jain A.K., Bhatnagar D. **Study of events leading to cellular Senescence to Human Mammary epithelial cancer cells in vitro**. *Indian. J. Cancer*. 38: 103-116, 2001
24. **Saxena S**., Beena KR, Bansal A, Bhatnagar A. **Emperipolesis: Significance of an unusual phenomenon in common breast malignancy**. *Acta Cytologica.* 46: 883-886, 2002
25. **Saxena S**, Szabo C, Barjhoux H, Chopin S, Siniliniova O, Lenoir G, Goldgar D, Bhatnagar D**. BRCA 1 and BRCA 2 in Indian Breast Cancer Patients.** *Human mutation.* 20 (6): 473-74, 2002.
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27. Bharat R, Burra U, Vidyadharan G, **Saxena S**. **Morphogical spectrum of cysticercus cellulose on cytology in case of malnutrished child**. *J Cytol*. 21 (2): 95-06, 2004
28. **Saxena S,** Bansal A, Mohil R S, Bhatnagar D. **Metaplastic carcinoma of the breast-A rare breast tumor.** *Ind J Pathol and Microbiol.* 47(2): 217-220, 2004
29. **C**hintamani, Shankar M, Singhal V, Singh J P, **Saxena S**. **Squamous cell carcinoma developing in the scar of fournier's gangrene-case report.** *BMC Cancer*. 4:16, 2004.
30. Bharat R, **Saxena S,** Burra U. **Fine needle aspiration cytology of Dermato fibrosarcoma protuberans**. *J Cytol*. 21(3), 2004
31. **C**hintamani, Singhal V, Singh J P, Bansal A, **Saxena S**, Lyall A. **Is drug induced cytotoxicity a good predictor of response to new adjuvant chemotherapy in breast cancer? A prospective clinical study.** *BMC Cancer*. Aug 13; 4(1): 48, 2004.
32. Mukherji A, Madholia V, Malhotra S, Singh P, Rekhi B, **Saxena S**, Aggarwal Y, Bhowmik K.T. **Multiple Myeloma Of The Breast –An Unusual Case Of Multiple Myeloma Of The Breast With Pathological Fractures Of Humerus and Femur**. *Jr. Of Clinical Radiotherapy and Oncology.4 (4):27-30, 2004.*
33. Mohanti. N.K, **Saxena . S**, Goyal .N.K, Singh. U.P, Arora .R.P **Delayed Cystectomy for T1G3 TCC of Urinary Bladder Managed initially by TURBT & Intravesical Immunotherapy (BCG+Interferron)—Rationale & our results.** *Indian Journal of Urology, 2004, vol. 20: 2*.
34. **C**hintamani, Singhal V, Singh J P, Bansal A, **Saxena S**. **Half versus full vacuum suction drainage after modified radical mastectomy for breast cancer-A prospective randomized clinical trial** (ISRCT N24484328). *BMC Cancer*. 5:11, 2005**.**
35. Rekhi B, Bansal A, Bhatnagar D, Bhatnagar A, **Saxena S. Cytomorphological study of soft tissue neoplasms: role of fluorescent immunocytochemistry in diagnosis** *Cytopathology, 16(5) :219-26, 2005.*
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presentations with cytologic and Ultrastructural findings. *Indian J Cancer. ,42(2):99-101,2005*

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40. N.K.Mohanty, **Sunita Saxena**, Uday Pratap Singh, Neeraj K.Goyal, R.P.Arora "Lycopene **as a chemoprevetvive agent in the treatment of High Grade Prostate Intraepithelial Neoplasia.**" *Urol Oncol. 2005 Nov-Dec; 23(6):383-5.*
41. Chintamani, Jai Prakash Singh, Mahesh K Mittal, **Sunita Saxena**, Anju Bansal, Ashima Bhatia, Pranjal Kulshreshtha **The role of P--glycoprotein expression in predicting response to neoadjuvant chemotherapy in breast cancer-a prospective clinical study.** *World Journal of Surgical Oncology* 2005 **3**:61
42. **Sunita Saxena** ; Bharat Rekhi ; Anju Bansal ; Ashok Bagga ; Chintamani C and N.S.Murthy: **Clinico-morphological patterns of Breast Cancers Including family history in a Delhi hospital, India- A Cross-sectional study** *World Journal of Surgical Oncology 2005, **3:**67*
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44. **Sunita Saxena**, Usha Agrawal, Abhilasha Agarwal*, Saurabh Verma, NS Murthy*, NK Mohanty **"Adjuvant Intravesical Therapy Based on In Vitro Cytotoxicity Assay In Management Of Superficial Transitional Cell Cancer of Urinary Bladder"** *BJU International 2006 Vol. 98 (5), 1012*
45. Bharat Rekhi, **Sunita Saxena "New Pot-pourri of Markers related to Invasive Breast Cancer"** *JIMSA 2006 19(1), Jan-Mar.*
46. Rekhi B, **Saxena S "Cytomorphology of Basal Cell Type Of Solid Ameloblastoma-A Case Report"** *J Of Cytology 2006;23:83-85*
47. **Sunita Saxena**, Anurupa Chakraborty, Mishi Kaushal Sanjeev Kotwal, Dinesh Bhatnagar, RS Mohil, Chintamani Chintamani, AK Aggarwal, Veena Sharma, PC Sharma, Gilbert Lenior and David Goldgar, Csilla Szabo **Contribution of germline BRCA1 and BRCA2 sequence alterations in to breast cancer in Northern India.** *BMC Medical Genetics 2006, 7:75.*
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49. Singh A, **Saxena S**. **"Infiltrating Duct Carcinoma Of Breast, Metastatic to Axillary Lymph Nodes Harbouring Primary Tuberculous Lymphadinitis"** *Pathology Oncology Research,12(3),2006*

50. Singh Avninder, Amar Bhatnagar, Usha Agrawal and **Sunita Saxena**. **Isolated splenic metastasis from colorectal mucinous carcinoma: a case report** *International Journal of Gastrointestinal Cancer 2006;37(2-3):98-101*

51. N S Murthy, Usha K Burra, K Chaudhry, and **S Saxena" Trends in incidence of breast cancer-Indian Scenario".** *Europian Jr. Of Cancer Care. doi:10.1111/j.1365-2354.2006.*

52. Anurupa Chakraborty[1], N.S. Murthy[2], Chintamani[3], D Bhatnagar[3], R.S. Mohil[3], A. Bhatnagar[3], P.C. Sharma[4]**, Sunita Saxena[1] CYP 17 gene polymorphism and its association with high-risk North-Indian breast cancer patients"** *Journal of Human Genetics 52(2):159-165,2007*

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54. Chintamani, Binita P Jha, Anju Bansal, **Sunita Saxena** and Dinesh Bhatnagar **The expression of mismatched repair genes and their correlation with clinicopathological parameters and response to neo-adjuvant chemotherapy in breast cancer** *International Seminars in Surgical Oncology.20074:5.*

55. Chintamani, Pranjal Kulshreshtha, Nidhi Sugandhi, Anju Bansal, Dinesh Bhatnagar and **Sunita Saxena. Is an aggressive approach justified in the management of an aggressive cancer-the squamous cell carcinoma of thyroid?** *International Seminars in Surgical Oncology* 2007, 4:8 doi:10.1186/1477-7800-4-8

56. Chintamani, Vinay Singhal, Anju Bansal, Dinesh Bhatnagar and **Sunita Saxena. Isolated colostomy site recurrence in rectal cancer - two cases with review of literature** *World Journal of Surgical Oncology 2007* ***5****:52*

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59. Sharma, M., Chintamani, Saxena, S. and Agarwal, U. **Squamous cell carcinoma arising in unilateral Warthin's tumor of parotid gland.** *J Oral Maxillo Facial Pathol 12 (2008).*

60. Anurupa Chakraborty[1], A.K Mishra[1], Abha Soni[1], Thodum Regina[1], D Bhatnagar,[2] A Bhatnagar[2], Chintamani,[2] Sunita Saxena[1] **VDR gene polymorphism(s) and breast cancer risk in North Indian Population.** *Cancer Detection and Prevention* **32** (2009) pp. 386-394

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