

CARACTERIZAÇÃO DA INTERAÇÃO DOS BACTERIOFAGAS
T4, M13, E MS2 COM CÉLULAS EPITELIAIS PROSTÁTICAS
TUMORIAIS (LNCAP E PC3): ATIVAÇÃO DAS VIAS DE
PROLIFERAÇÃO, SOBREVIVÊNCIA
E MORTE CELULAR

SWAPNIL GANESH SANMUKH

*Tese apresentada ao Instituto de Biociências,
Câmpus de Botucatu, UNESP, para obtenção do
título de Doutor no Programa de Pós-
Graduação em Biologia Geral e Aplicada, Área
de concentração Biologia Celular Estrutural e
Funcional.*

Nome do orientador Sérgio Luis Felisbino

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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



UNIVERSIDADE ESTADUAL PAULISTA

"Julio de Mesquita Filho"

INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

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Caracterização da interação dos bacteriófagos T4, M13, e MS2 com células epiteliais prostáticas tumoriais (LNCaP e PC3): ativação das vias de proliferação, sobrevivência e morte celular.

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DEDICATION

This thesis work is dedicated to my mother, Dr. Usha Ganesh Sanmukh who is my constant source of inspiration, support, and encouragement during all the challenges of my life. I am also very grateful to my late grandparents Mr. Bhupal Aba Kamble (01-01-1935 to 17-01-2005) and Mrs. Indumati Bhupal Kamble (15-04-1934 to 26-02-2013), who brought me up well in an educated environment, which will be guiding and inspiring me through there teachings in every walk of life. I also like to thank all my family members from India who directly or indirectly helped me throughout my doctoral journey. Their unconditional love and support have helped me to work hard for the things that I aspire to achieve.

“My brain is only a receiver, in the Universe, there is a core from which we obtain knowledge, strength, and inspiration. I have not penetrated into the secrets of this core, but I know that it exists”

— Nikola Tesla

“It’s not what you achieve, it’s what you overcome that’s what defines your career”

— Carlton Fisk

DEDICATION

My poem dedicated to My Mother.....

*Oh, my lovely mother, to whom I never prefer,
For her, I was a duffer, who does nothing but to make her suffer!*

*All the times, when I saw her eyes,
Full of delight, full of shine,
Saying something, which I never mind,
But for her, I was never so kind!
She has faced the whole world with ease,
For none, she stopped, for none she seized,
But for us, she was always please,
Oh, my mother, you are all we need.*

- By Swapnil Sanmukh

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LIST OF ABBREVIATIONS

- AAVP:** Adeno-Associated Virus/Phage;
- AD:** androgen dependent cells (AD);
- AI:** androgen-independent (AI);
- APHA:** American Public Health Agency;
- AR:** Androgen receptor;
- ATCC:** American Type Culture Collection;
- BPH:** Benign prostatic hyperplasia (BPH);
- CFU:** Colony forming unit;
- CK8:** Human cytokeratin 8 gene is regulated by wild-type p53;
- CTA:** Clinical Trial Application;
- ECM:** Extracellular Matrix;
- EGFR:** Epidermal growth factor receptor;
- EMA:** European Medicines Agency;
- EMT:** Epithelial mesenchymal transition;
- EPCA:** Early Prostate Cancer Antigen;
- ERK:** Extracellular signal Regulated Kinases (MAP kinases or MAPK: mitogen activated protein kinase);
- FAK:** Focal Adhesion Kinase (FAK), also known as PTK2 protein tyrosine kinase 2 (PTK2);
- FDA:** The Food and Drug Administration (FDA or USFDA);
- GRP78:** Glucose Regulated Protein;
- GSTP1:** glutathione S-transferase pi 1 (human) or Glutathione S-transferases (GSTs)
- HAP1 Phage:** A T4 Sub-Strain with A High Affinity to Melanoma Cells;
- HRP:** Horseradish Peroxidase;
- HSP27:** Heat Shock Protein 27;
- HSP70:** Heat Shock Protein 70;
- HSP90:** Heat Shock Protein 90;
- hTERT:** Human telomerase reverse transcriptase
- IAP:** Inhibitor of apoptosis genes;
- IF:** Interferon;
- IGF:** Insulin-like growth factor;
- IGF-1R:** Insulin-like growth factor 1 receptor;

- IGF-2R:** Insulin-like growth factor 2 receptor;
- IL:** Interleukin;
- IND:** Investigational New Drug;
- ITGAV:** Integrin Alpha V;
- ITGA5:** Integrin Alpha 5;
- ITGB1:** Integrin Beta 1;
- ITGB3:** Integrin Beta 3;
- ITGB5:** Integrin Beta 5;
- KGD Motifs:** Tripeptide Lysine-Glycine-Asparagine Motifs;
- LAP:** latency associated peptide;
- LNCaP:** Lymph Node Carcinoma of the Prostate;
- MAPK/ERK pathway:** It is also known as the Ras-Raf-MEK-ERK pathway, are chain of proteins that communicates signals from cell surface receptor to the DNA in the nucleus of cell;
- MMPs:** Matrix Metallo-Proteinases (MMPs), also known as Matrixins;
- MTCC:** Microbial Type Culture Collection;
- mTOR:** Mechanistic target of rapamycin (mTOR), (formerly mammalian target of rapamycin before it was recognized to be highly conserved among eukaryotes);
- MYC oncogenes:** Family of retrovirus-associated DNA sequences (myc) originally isolated from an avian myelocytomatosis virus;
- NE:** Neuroendocrine cells;
- PBS:** Phosphate Buffer Saline;
- PC3:** Prostate cancer cell line;
- PCa:** Prostate Cancer;
- PCA3:** prostate cancer antigen 3 (PCA3);
- PCC:** Progression markers in cell cycle;
- PFU:** Plaque forming unit;
- PGC-1alpha:** Peroxisome proliferator-activated receptor gamma co-activator 1alpha;
- PHF8:** PHD finger protein 8;
- PI3K:** Phosphatidylinositol-4, 5-bisphosphate 3-kinase;
- PIN:** Prostatic Intraepithelial Neoplasia;
- PKB:** Protein kinase B (also known as AKT);
- PMN:** Polymorphonuclear Leukocytes;
- PSA:** Prostate Serum Antigen;
- PSMA:** Prostate-specific membrane antigen (PSMA);

LIST OF ABBREVIATIONS

RAF: Rapidly Accelerated Fibrosarcoma;

RAS: Ras, a small GTP-binding protein, is an important component of the signal transduction pathway used by growth factors to initiate cell growth and differentiation;

RGD: Tripeptide Arginine-Glycine-Asparagine;

s-ARU: Semi-adherent relative upsurge;

SCNC: Small cell (neuroendocrine) carcinoma;

STAT: Signal transducer and activator of transcription protein family;

Thps: Tumor Homing Peptides;

Tsips: Tumor Specific Internalizing Peptides;

TPTZ: 2,3,5-Triphenyltetrazolium chloride;

USFDA: United States Food and Drug Administration;

VEGF: Vascular endothelial growth factor

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RESUMO

Sanmukh SG. **Caracterização da interação dos bacteriófagos T4, M13 e MS2 com células epiteliais prostáticas tumorais (LNCaP e PC3) : ativação das vias de proliferação, sobrevivência e morte celular** [Tese]. Botucatu, São Paulo: Instituto de Biociências, Universidade Estadual Paulista – UNESP, 2018.

O câncer de próstata (PCa) é o segundo tipo de câncer mais frequente e tem a segunda maior taxa de morbidade e mortalidade entre os homens. A cultura de células prostáticas LNCaP e PC3 tem sido utilizada para investigar possíveis alvos e vias de sinalização celular importantes para o crescimento e progressão do CaP. Trabalhos anteriores do nosso laboratório demonstraram que a presença de fibronectina no meio de cultura altera significativamente o padrão de expressão gênica dessas células. Também, bacteriófagos recentemente usados como agentes terapêuticos no tratamento de vários tipos de câncer, diretamente ou portadores de moléculas antitumorais, incluindo câncer de próstata, foram relatados. Estudos anteriores mostraram que bacteriófagos podem interagir com proteínas de membrana de células de mamíferos, incluindo integrinas que reconhecem sequências de RGD em proteínas virais, bem como fibronectina e outras moléculas da matriz extracelular. O objetivo deste projeto foi caracterizar a interação de três bacteriófagos com as duas linhagens celulares epiteliais prostáticas LNCaP e PC3. Estas duas linhas celulares foram cultivadas em seus meios de cultura, nos quais foram adicionados bacteriófagos com concentrações definidas. As células foram coletadas após 24 horas de tratamento. A expressão gênica de genes relacionados as vias integrinas ITGA5, ITGAV, ITGB1, ITGB3, ITGB5, AKT, PI3K, MAPK1, MAPK3, HSP27, HSP90, receptor de androgênio AR, STAT3, PGC1A foi analisada por qPCR. A ativação da via AKT/PI3K foi analisada também por western blotting. Células PC3 foram expostas aos bacteriófagos T4 e M13 e foram analisadas por microscopia de luz convencional e por microscopia de varredura. Nas células LNCaP, o bacteriófago M13 regulou negativamente genes ITGA5, ITGAV, ITGB1, ITGB3, ITGB5, MAPK1, MAPK3, HSP90, TBP, PSA e positivamente

genes AKT, PI3K, HSP27, AR, STAT3, PGC1A genes; o bacteriófago MS2 regulou negativamente genes ITGA5, ITGAV, ITGB3, ITGB5, MAPK1, MAPK3, HSP90, PI3K, PSA e positivamente genes ITGB1, AKT, HSP27, AR, TBP, STAT3, PGC1A genes; o bacteriófago T4 regulou negativamente genes ITGA5, ITGB3, ITGB5, HSP27, HSP90, TBP, PGC1A, PSA e positivamente genes ITGB1, ITGAV, AKT, PI3K, AR, MAPK1, MAPK3, STAT3 genes. Já para as células PC3, os bacteriófagos M13 e T4 regularam negativamente genes HSP27, HSP90, AR e positivamente os genes ITGA5, ITGAV, ITGB3, ITGB5. Microscopicamente foi possível observar a interação dos bacteriófagos T4 e M13 com a superfície celular das células PC3 e que esta interação com o bacteriófago M13 reduz a migração celular. Nossos resultados sugerem que os 3 bacteriófagos podem alterar a expressão de genes relacionados à adesão e proliferação celular, de uma maneira célula específica e podem funcionar como nanopartículas com potencial terapêutico para o tratamento de diferentes tipos de câncer, incluindo o câncer de próstata.

Palavras-chave: Câncer, próstata, bacteriófago, expressão gênica, integrinas, cultura célula,

ABSTRACT

Sanmukh SG. **Characterization of the interaction of bacteriophage T4, M13 and MS2 with tumoral prostatic epithelial cells (LNCaP and PC3): activation of proliferation pathways, survival and cell death** [Tese]. Botucatu, Sao Paulo: Instituto of Biosciences, State University of Sao Paulo – UNESP, 2018.

Prostate cancer (PCa) is the second most frequent type of cancer and has the second highest rate of morbidity and mortality among men. The LNCaP and PC3 prostate cell culture has been used to investigate possible targets and cell signaling pathways important for the growth and progression of PCa. Previous work from our laboratory has demonstrated that the presence of fibronectin in the culture medium significantly alters the gene expression pattern of these cells. Also, bacteriophages recently used as therapeutic agents in the treatment of various types of cancer, directly or carriers of antitumor molecules, including prostate cancer, have been reported. Previous studies have shown that bacteriophages can interact with mammalian cell membrane proteins, including integrins that recognize RGD sequences in viral proteins, as well as fibronectin and other extracellular matrix molecules. The objective of this project was to characterize the interaction of three bacteriophages (T4, M13, and MS2) with the two prostatic epithelial cell lines LNCaP and PC3. These two cell lines were cultured in their culture media, in which bacteriophages with defined concentrations were added. Cells were collected after 24 hours of treatment. The gene expression of genes related to the integrin pathways *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *AKT*, *PI3K*, *MAPK1*, *MAPK3*, *HSP27*, *HSP90*, androgen receptor *AR*, *STAT3*, *PGC1A* was analyzed by qPCR. Activation of the AKT / PI3K pathway was also analyzed by western blotting. PC3 cells were exposed to T4 and M13 bacteriophages and analyzed by standard light microscopy and scanning microscopy. In the LNCaP cells, the bacteriophage M13 regulated negatively genes *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *MAPK1*, *MAPK3*, *HSP90*, *TBP*, *PSA* and positively *AKT* genes, *PI3K*, *HSP27*, *AR*, *STAT3*, *PGC1A* genes; the bacteriophage MS2 negatively regulated genes *ITGA5*, *ITGAV*, *ITGB3*, *MAPK1*, *MAPK3*,

ABSTRACT

HSP90, *PI3K*, *PSA* and positively genes *ITGB1*, *AKT*, *HSP27*, *AR*, *TBP*, *STAT3*, *PGC1A* genes; the bacteriophage T4 regulated negatively genes *ITGA5*, *ITGB3*, *ITGB5*, *HSP27*, *HSP90*, *TBP*, *PGC1A*, *PSA* and positively genes *ITGB1*, *ITGAV*, *AKT*, *PI3K*, *AR*, *MAPK1*, *MAPK3*, *STAT3* genes. As for PC3 cells, M13 and T4 bacteriophages negatively regulated *HSP27*, *HSP90*, and *AR* genes and positively regulated the *ITGA5*, *ITGAV*, *ITGB3*, *ITGB5* genes. Microscopically it was possible to observe the interaction of T4 and M13 bacteriophages with the cell surface of PC3 cells and that interaction with bacteriophage M13 reduces cell migration. Our results suggest that the 3 bacteriophages can alter the expression of genes related to cell adhesion and proliferation in a cell specific manner and may function as nanoparticles with therapeutic potential for the treatment of different types of cancer, including prostate cancer.

Key words: Cancer, prostate, bacteriophage, gene expression, integrins, cell culture.

1 INTRODUCTION

1.1 Prostate Cancer

In men, prostate glands are walnut shaped organ located below the bladder surrounding urethra. It's a gland exclusive of mammals. In humans, the prostate has four zones, central, transitional, peripheral and fibromuscular (Verze et al., 2016). The function of prostate glands is to secrete an alkaline fluid that forms a part of seminal fluid which is important for male fertility in many ways, such as sperm maturation, motility, clotting and liquefaction (Verze et al., 2016). Prostate gland is prone to develop several diseases, such prostatitis, benign prostatic hyperplasia and prostate cancer (Verze et al., 2016).

Prostate cancer has been reported to affect a large male population in the western world. The latest USA and global estimate pointed to prostate cancer (PCa) being the most common cancer in men and the second leading cause of cancer deaths in men; approximately 75% of diagnosed cases in the world occur in developed countries. The worldwide incidence rate has increased by 25% in recent decades, with the highest observed in Australia, New Zealand, Western Europe and North America (Torre et al., 2016; Siegel et al., 2018). In Brazil, the estimate for the year 2018 was 68,220 new cases of prostate cancer, behind only projections of incidence for non-melanoma skin cancer (INCA, <http://www.inca.gov.br/estimate/2018>). There are global estimates, alarming approximately 1.7 million new cases and 500,000 deaths by the year 2030 (Center et al., 2012).

Prostate cancer is very complex disease based on its biological, hormonal and genetic nature and based upon its histopathological heterogeneity, with different factors contributing to

its initiation and progression (Shoag and Barbieri 2016; Boyd et al., 2012; Ross-Adams et al., 2015; Wei et al., 2017; Tolkach and Kristiansen, 2018).

Circulating androgens are essential for normal development of the prostate gland as well as in the initial development of PCa through their interactions with AR (Hsing et al., 2002). As demonstrated by Huggins and colleagues in the 1940s, the removal of testicular androgens by surgical or chemical castration leads to the regression of PCa (Huggins et al., 1941; Huggins & Hodges, 1941). However, androgen deprivation is usually associated with recurrence of PCa, monitored by elevated PSA levels, and this recurrent disease is called castration-resistant PCa (Tan et al., 2015). This term castration resistance has replaced the term androgen independent, as it became apparent that the advanced PCa remains dependent on AR function by intracrine and paracrine mechanisms (Makarov et al., 2009; Schiffer et al., 2017). This progression status of the disease are also related with changes in the androgen receptor (AR) function by amplifications, mutations and activation without ligand (Tan et al., 2015).

In contrast, the increased incidence of PCa in the world population may reflect increased life expectancy, improvements in the health information system, and screening practices using the PSA test. PSA is a serine protease from the kallikrein family that is produced by prostatic epithelial cells and secreted into the glandular lumen. However, when there is the destruction of the glandular architecture due to the presence of adenocarcinoma, PSA is released into the blood (Lilja et al., 2008). PSA is the most used serum marker in the diagnosis, prognosis, and monitoring of treatments for PCa. Men with elevated serum PSA above 4ng / ml will typically undergo a biopsy to assess the presence of PCa. However, recent studies have shown that approximately 15% of men with PSA <4.0 ng / ml had PCa (Thompson et al., 2004), suggesting that lower amounts (e.g., 2.5 ng / ml) can be used, especially among younger patients (younger than 40 years and with a family history of risk). As PSA levels may be altered due to other factors, such as the presence of benign prostatic hyperplasia (BPH) and prostatitis, methods that

refine the accuracy of PSA (speed, density, age-adjusted PSA, molecular forms of PSA) also have been used, according to the clinical context (Greene et al., 2013).

The introduction of the PSA test to monitor the treatment of PCa also led to the widespread diffusion of its use as a screening test among asymptomatic men (Esserman et al., 2009). Although PSA is used to evaluate the progression, response to treatment and disease recurrence after prostatectomy, it does not represent as a specific biomarker for PCa and other non-invasive diagnostic markers have been searched (Prensner et al., 2012).

1.2 Non-PSA-based diagnostic test for Prostate cancer

The prostate cancer gene 3 (PCA3) is a long non-coding RNA that is high in > 90% of PCa tissue but not in normal tissue or BPH (Bussemakers et al., 1999). The high sensitivity and specificity of PCA3 have been used for its evaluation by non-invasive methods, such as assays to detect it in urine samples from patients, which contains cells released from the prostate during urination (Merola et al., 2015). Currently, a clinical-grade clinical trial is available based on transcription followed by amplification (Hessels & Schalken, 2009). In 2012, PCA3 was approved by the FDA (Federal Drug Administrator of the United States of America) as a test for the PCa to define negative prostate biopsies. In addition to PCA3, early prostate cancer antigen (EPCA - Early Prostate Cancer Antigen) has also been introduced as a serum marker for detection of PCa (Dhir et al., 2004, Zhao et al., 2010; Zhao et al., 2012). Although this two new markers, PSA analysis is still the main diagnostic biomarker in use.

The histopathological staging of PCa according to the Gleason grade or standard is based on glandular histoarchitecture and classifies tumors from 1 to 5 (from most to least differentiated). In the end, a Gleason score is given which is resulting from the sum of the two most predominant patterns in prostate tissue, ranging from 2-10 (Mellinger et al., 1967; Epstein, 2010). The Gleason Scores of 7, 8, 9 and 10 are associated with more aggressive disease and

poorer prognosis of healing, while Gleason Scores of 2-6 are considered better prognosis (Calabro & Sternberg, 2007).

The progression from preclinical to clinical disease is quite slow and silent, and occurs through a series of defined stages, beginning with prostatic intraepithelial neoplasia (PIN) and progressing to micro-invasive adenocarcinoma, invasive and metastatic adenocarcinoma, androgen-dependent and then androgen-independent metastatic adenocarcinoma (Nelson et al., 2003; Sakr et al., 1994). In contrast to the histological appearance of the disease, clinical signs usually do not appear before 50 years of age (Rittmaster et al., 2009). The clear majority of PCa correspond to acinar adenocarcinoma expressing the androgen receptor (AR), while other categories of PCa, such as ductal adenocarcinoma, mucosal carcinoma, and others are extremely rare (Grignon, 2004). The most significant histological variant may be neuroendocrine PCa, which is also generically classified as small cell carcinoma and represents <2% of PCa cases (Grignon, 2004).

Notably, focal areas of neuroendocrine differentiation are commonly seen in adenocarcinoma of the prostate, particularly the following recurrence after prostatectomy and androgen deprivation therapy (Yuan et al., 2007; Komiya et al., 2005). The expression of Chromogranin neuroendocrine cell marker is associated with the development of castration-resistant tumors, and shorter time to recurrence of the disease (Kokubo et al., 2005; Berruti et al., 2007). This prevalence of neuroendocrine differentiation after relapse may be due to the lack of AR expression in these cells, which are inherently castration-resistant.

Despite the advances in early detection capacity of PCa, integrating PSA dosage with rectal examination and imaging systems, the main challenge is the difficulty in distinguishing in the aggressive tumors in patients with low PCa Gleason score (Choudhury et al., 2012).

1.3 Prognostic markers

As mentioned earlier, as important as diagnosing PCa, it is also imperative to distinguish between aggressive and non-aggressive subtypes. Until recently, the available genetic alterations did not provide greater prognostic advantage than determining the Gleason score (lower score better the prognosis), PSA levels (lower levels correlate with a better prognosis) and tumor status (confined body correlates with better prognosis) (True et al., 2006; Makarov et al 2009). However, with advances in genotyping technologies, analysis of transcriptomes by sequencing or microarray, together with a better understanding of the mechanisms involved in PCa by means of experimental and computational methods, new markers associated with initiation, progression and response / resistance to treatments. Thus, integrated analysis of gene expression, copy number changes, and polymorphisms has provided a unified approach to distinguish subtypes of PCa and characterize patient's outcome (Taylor et al., 2010; Markert et al., 2011; Choudhury et al., 2012; Ross-Adams et al., 2015).

At the time of diagnosis, prostate tumor cells contain several somatic mutations, deletions and gene amplification, chromosomal rearrangements, and changes in DNA methylation, which are probably accumulated over the decades. Contrarily, to the other types of cancer, a surprising heterogeneity of genetic and epigenetic alterations has been described in different cases, in different lesions in the same case and in different areas within the same lesion, making it even more difficult to determine prognostic markers of PCa (Elo & Visakorpi, 2001; Taylor et al., 2010.). In addition, additional somatic genomic changes seem to arise with the progression of PCa (Abate et al., 2003; Shen & Abate-Shen, 2010). The epigenetic silencing of gene expression by methylation of the GSTP1, reducing the expression of a glutathione, a key enzyme in response to oxidative stress (Nelson et al., 2003) and altered expression of microRNAs, such as miR-221 / 222-25 that negatively regulates expression of p27 (Delella et al., 2012).

Gene expression analysis has been an important tool for understanding the behavior of tumors since the mRNA levels of several genes at one time may reflect the phenotype or the

cancer state rather than specific genetic variants or other isolated structural changes. In this sense, gene expression signatures have been successfully applied to define subclasses of breast cancer with different biological behaviors and responses to therapies. Diagnostic tests based on gene expression under the trade names of Oncotype Dx (Genomic Health, Inc., Redwood City, CA, USA) and Mamma Print (Agendia, Irvine, CA, USA) are currently in clinical use to assess the risk of relapse and predicting the benefits of adjuvant chemotherapy in patients with breast cancer located in estrogen receptor positive and node-negative (Oakman et al., 2010).

Several groups have published gene expression signatures of primary PCa tumors that correlate with worse prognosis in retrospective analyses (Cancer Genome Atlas Research Network, 2015). However, the list of subscriptions generated in these genes usually does not overlap between studies and no set of genes was further validated for clinical use (Cooper et al., 2007; Peng et al., 2014). Among the more recent include the groups accessed the gene expression levels related to stem cell phenotype (Glinsky et al., 2005; Markert et al., 2011) or increased progression markers in cell cycle (PCC) (Cuzick et Al., 2011), which potentially reflect a more aggressive state of the disease and worse prognosis of survival after anti-androgen therapy. In this regard, a panel that accesses the expression of 46 genes involved in PCC is being commercially developed by Myriad Genetics (Salt Lake City, UT, USA) under the trade name Prolaris. However, more studies are needed to validate prognostic markers in a standardized way.

1.4 Integrins and Cancer

Since this work is focused on the interaction of bacteriophages with cell surface proteins, it is important to describe the major class of ligand protein mainly integrins. The integrins are important component of cell membrane which helps cells to interact with their extracellular matrix (ECM) microenvironment. Integrins detect both physical and chemical signals from the

matrix by multiprotein plasma membrane complexes and transmit this information's by cytoskeleton and signalling proteins for the proper functioning of both nuclei and cytosol as represented in figure 2 (Streuli, 2016).

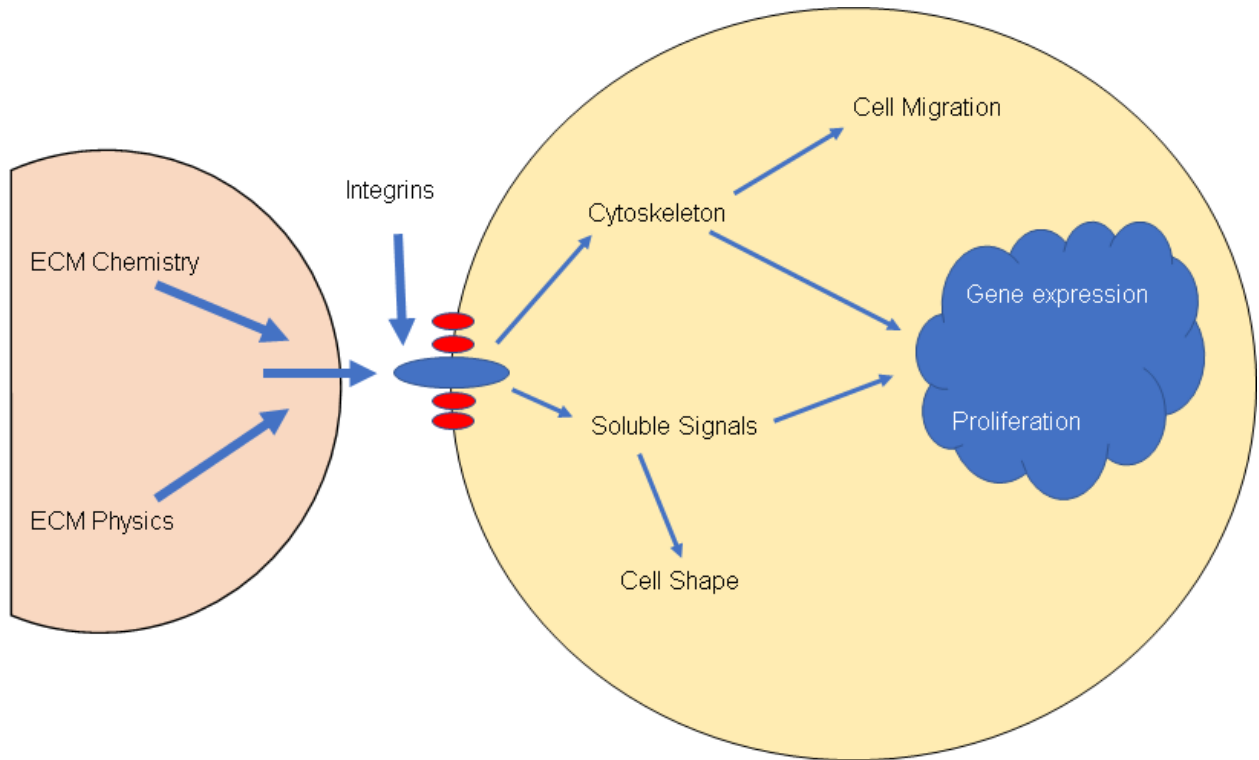


Figure 1. Diagrammatic representation of how integrins mediated interaction between extracellular matrix and cell components while regulating cellular architecture and functions. (Adapted from Streuli 2016).

Considering the importance of integrins in cancer, Nieberler et al. in 2017, have provided a magnificent account of role of integrins in different steps of cancer progression, which are as follows;

- (i) Cell adhesion is regulated by recognizing specific RGD (arginine-glycine-aspartic acid motif) ligands within the extracellular matrix through integrins (Nieberler et al., 2017).
- (ii) The $\alpha\beta3$, $\alpha\beta6$, and $\alpha\beta8$ integrins may induce cell proliferation through RGD with latency associated peptide (LAP) in TGF- β binding to integrins (Nieberler et al., 2017).

(iii) Overexpression of integrins can help cancer cells to bind extracellular matrix components and inhibit apoptosis and/or anoikis of invasive carcinoma in mesenchymal tissue (Nieberler et al., 2017).

(iv) During tumor growth, the expression of factors associated with hypoxia and VEGF can result in integrin mediated vessel germination promoting angiogenesis (Nieberler et al., 2017).

(v) The epithelial mesenchymal transition (EMT) like alteration is responsible for integrin switching from $\alpha\beta1$ and/or $\alpha\beta5$ to $\alpha\beta6$, which promotes migration across basement membrane, allowing downregulation of E-cadherin and other cell adhesion molecules on one hand and enhancing cancer progression and metastasis on the other (Vellon et al., 2007; Nieberler et al., 2017).

Integrins regulate angiogenesis during tumor formation and growth process (Hynes 2002; Hynes 2007; Sakamoto et al. 2008). It is reported through treatment of a PC3 xenograft through $\alpha\beta3$ antagonist (cyclic RGD) can induce and increase anti-angiogenic and anti-tumor effects (Abdollahi et al. 2005). A human antibody CNTO95 can recognize integrin $\alpha\beta$ and binds to both integrin $\alpha\beta3$ and $\alpha\beta5$ (Tripathi et al., 2004). It is also reported to inhibit adhesion and migration as well as the growth of human melanoma tumours in nude mice effectively (Suyin et al., 2013).

The $\alpha\beta3$ integrin is linked to the Ras-ERK signalling pathway, as activation of MAP kinase is adhesion-dependant which can be of great important in regulating cell proliferation through integrins (Wary et al., 1996, Kumar 1998; Adachi et al., 2001). It has also been reported that in human embryonic kidney epithelial cells treatment of $\alpha\beta3$ antagonists disrupted adhesion to the VN matrix and induced apoptosis (Brassard et al., 1999). Also, adhesion mediated by integrins leads to activation of PI3K independently (Adachi et al., 2001) and thereby activates AKT leading to suppression of apoptosis and other biological effects (Khwaja, 1999). The AKT phosphorylates Bcl-XL, dissociating Bad which further act as a suppressor of apoptosis (Franke and Cantley, 1997, Adachi et al., 2001).

As integrins regulate matrix metalloproteinases, they can degrade or remodel extracellular matrix which can affect various cancer processes like migration, invasion and metastasis (Naci et al., 2015; Borrirukwanit et al., 2014; Missan et al., 2015; Schlomann et al., 2015; Blandin et al., 2015). The $\beta 1$ or $\beta 4$ are responsible for cell adhesion to collagen or laminin. $\beta 1$ Integrins are overexpressed in several solid tumors in comparison to control tissues. Targeting αv and $\beta 1$ integrins through nanoparticle with siRNA has decreased the phosphorylation of c-Met and of EGFR during liver regeneration, which is important for cell proliferation (Morozevich et al., 2012; Bogorad et al., 2014; Speicher et al., 2014). Overexpression of $\alpha 5$ in p53 wild-type liver tumor increased resistance to therapies (Janouskova et al., 2012) and targeting of $\alpha 5$ integrin by miR-26a or inhibiting $\alpha v\beta 3/\beta 5$ integrin through cilengitide promote anoikis (Silginer et al., 2014).

Inhibition or downregulation of $\alpha 2\beta 1$ integrin can avoid chemoresistance against doxorubicin as reported in leukemia (Naci et al., 2012). Also, downregulating $\alpha v\beta 3$ integrin/KRAS as well as $\beta 1$ integrin in solid tumors can be useful for EGFR targeted therapies (Seguin et al., 2014; Huang et al., 2011; Morello et al., 2011; Eke et al., 2013; Kanda et al., 2013; Blandin et al., 2015).

In other cancers, such as gastric, FAK signalling is blocked through reduced $\beta 1$ integrin expression (Cheng et al., 2015). Most of the malignancies are attributed to hTERT (Human telomerase reverse transcriptase) and, curiously, telomerase is reported to promote cancer invasion by enhancing $\beta 1$ integrin in some cancers (Hu et al., 2015; He et al., 2016). The miR-25 interacting with αv has shown tumor suppressive effects in prostate cancer (Zhang et al., 2015; Zoni et al., 2015). The $\alpha v\beta 3$, $\alpha 2\beta 1$, and $\alpha 4\beta 1$ integrins are crucial in cancer cell progression to bone metastasis (Esposito and Kang, 2014). Moreover, higher expression of $\alpha 3\beta 1$ is associated with a higher reappearance of prostate cancer (Pontes-Junior et al., 2010). Zhang et al have reported that highly invasive PC3 cell line express $\alpha v\beta 3$ integrin; whereas, the LNCaP cell line

don't. Interestingly, the adherence and migration of PC3 cells on vitronectin, which is an $\alpha v\beta 3$ ligand indicates its possible metastasis location in bone and LNCaP cells cease to show such properties (Zheng et al., 1999; Nemeth et al., 2003). Hence, $\alpha v\beta 3$ is a mediator of prostate epithelial cell migration through increased tyrosine phosphorylation of focal adhesion kinase (FAK) making it a potential target in prostate cancer cell invasion and metastasis.

The upregulation of $\alpha 5\beta 1$ integrin in PC3 plays important role in the adhesion and spreading, similarly $\alpha v\beta 3$ integrin seems to regulate other receptors in the spreading process. Moreover, interaction between integrin and FN through RGD sequence restricted the process of cell adhesion and spreading (Stachurska et al. 2012). Out of known 24 human integrin subtypes integrin $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, and $\alpha IIb\beta 3$, recognize the tripeptide RGD motif within ECM proteins (Schittenhelm et al., 2013, Nieberler et al., 2017).

In case of cancer stem cells, integrin $\alpha v\beta 3$ and $\alpha 5\beta 1$ influences differentiation in different cancer stem cells with the help of other growth factors and such cells has increased expression of $\alpha v\beta 3$ on their membranes (Jordan and Guzman, 2006; Nieberler et al., 2017). Targeting $\alpha v\beta 3$ and other signaling molecules like K-Ras are under investigation to provide a solution for reoccurrence of cancer by resistance to treatment (Seguin et al., 2014). Whereas, increased expression of $\alpha v\beta 3$ is also reported to be a good indicator for chemosensitivity (Vellon et al., 2007; Nieberler et al., 2017)

Except $\alpha V\beta 1$, almost all the integrins are of cell signalling molecules which can be targeted by specific compounds and targeting $\alpha V\beta 1$ integrin has been reported to be a therapeutic application in fibrotic diseases (Reed et al., 2015; Kingwell, 2015). Sometimes the decrease in integrin signalling has been reported to induces autophagy until they reengage with the ECM (Lock and Debnath, 2008) whereas, loss of ECM adhesion can result in entosis (Overholtzer et al., 2007).

The suppression of $\alpha 3\beta 1$ reduces cyclooxygenase-2 gene expression which in response inhibits important tumor processes like tumorigenesis, invasion and metastasis (Mitchell et al., 2010). The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are important for tumor progression and metastasis because of their role in angiogenesis and promote cell survival (Malyankar et al., 2000; Erdreich-Epstein et al., 2000; Heß et al., 2014).

Also, $\alpha 5\beta 1$ integrin acts as an initiator for angiogenesis (Schaffner et al., 2013) whereas $\beta 1$ integrin is key regulator (Hakanpaa et al., 2015; Hongu et al., 2015; Vitorino et al., 2015; Yamamoto et al., 2015; Blandin et al., 2015). It has also been reported that Bevacizumab, which is a monoclonal antibody against VEGF-A in brain tumor has led to resistance due to overexpression of $\alpha 5\beta 1$ integrin and fibronectin. Hence, targeting $\beta 1$ integrin can increase the anti-VEGF-A effect of Bevacizumab (Carbonell et al., 2013; Jahangiri et al., 2014). Also, αv integrins have a very important role in prostate tumor survival in the bone (Bisanz et al., 2005). The intratumoral encapsulated liposomal-human αv -siRNAs have shown considerable reduction in PC3 growth and promote apoptosis of prostate tumor cells and McCabe *et al.* (2007) demonstrated that $\alpha v\beta 3$ integrin activation is essential for the locating bone-specific matrix proteins for metastasis along with $\alpha 2\beta 1$ (Hall *et al.* 2006; Blandin et al., 2015).

Recently, considering the importance of integrins in cancer progression, various patents have been reported for integrin inhibitors. The anti- αV integrin antibody (Patent: US 2017/0218070 A1) for the treatment of prostate cancer have been patented recently by Merck Inc. (Merck Patent GmbH, Darmstadt, Germany). The compounds comprising R-G-Cysteic Acid (US20130129621A1), for inhibiting integrins by avoiding cellular adhesion to RGD binding sites and thereby inhibiting angiogenesis in tumors and also through delivering other diagnostic or therapeutic agents to RGD binding sites in human or animal subjects. The inhibitor of $\alpha 7$ (WO2014072940A1) for the treatment, suppression and/or prevention of tumours. In a detailed review, Kapp et al., have provided details of patents awarded for integrin inhibitors for β -2, α -4,

α IIb β 3, α v β 3, α v β 5, α v β 6, and α 5 β 1 including another collagen binding integrins (Kapp et al., 2013).

1.5 Prostate cancer cell culture models

For the in-vitro studies, many cell culture models have been developed. The primary cultures which are finite, whereas permanent cell lines are derived from it for studying PCa in murine and human (Miki &, Rhim 2008). The possibility of working with human PCa cells in culture is a great advantage, mainly due to the acquired maturity of the three main cell lines, LNCaP, PC3, and DU-145. These strains are well described with respect to their methods of cultivation and other requirements (Culture media, additives, CO₂ and O₂ saturation, pH, spheres, surfaces of the plastics of the flasks) (Miki &, Rhim 2008).

In the present project, we chose to perform functional studies on two tumor cell lines with different characteristics and biology: the androgen-dependent LNCaP line and the androgen-independent PC3.

The PC3 line was isolated from a metastasis of a lumbar vertebra (Kaighn et al., 1979). The pathological diagnosis of primary and metastatic cancer was undifferentiated in such prostate adenocarcinoma. The cells grow attached to the culture flasks, as well as in three-dimensional frameworks. These cells are classically known to be androgen-independent, however, studies have shown these cells express some amount of androgen receptor mRNA and protein (Webber et al., 1997; Alimirah et al., 2006; Moroz et al., 2013).

The line LNCaP was isolated by Schuurmans and Mulder in 1977 and were derived from biopsy fragments of a metastasis located in the lymph nodes in 50 years old man (Horoszewicz et al., 1980, Horoszewicz et al., 1983; Webber et al., 1997). This prostate cancer that has metastasized had been diagnosed as moderately differentiated a year earlier. The mean cell

doubling time was determined to be between 60-72 hours, depending on the amount of fetal serum added to the culture medium. These cells have low anchorage potential and do not form a uniform monolayer in cell culture. They have cytoplasmic and nuclear androgen receptors, which stimulate their growth (Webber et al., 1997).

In previous studies by our group, we have demonstrated that these cell lines have different patterns of gene and protein expression when cultured in contact with extracellular matrix components, such as fibronectin (Moroz et al., 2013). We have shown that, in contact with the plastic substrate, these cells do not secrete detectable amounts of matrix metalloproteinases. However, when fibronectin is added to the medium, these cells express considerable amounts of MMP2 and MMP9. The mechanism for expression of these genes is related to the activation of the alpha5 / beta1 / FAK / PI3K / AKT / ERK integrin pathway, as described by Das et al. (2008). In this way, we are interested in exploring other changes in gene expression induced by binding of these cells to the extracellular matrix, such as the androgen receptor and IGF / IGF-1R / IGF-2R pathways.

1.6 Comparison between LNCaP and PC3

It has been well documented and reported by Tai et al. 2011, that normal prostate epithelium has luminal epithelial cells, basal cells and a small component of neuroendocrine (NE) cells that are scattered throughout the prostate. The PCs are adenocarcinomas when they show the absence of basal cells and uncontrolled proliferation cells along with glandular formation with the expression of androgen receptor (AR) and prostate-specific antigen (PSA) (Tia et al., 2011). The neuroendocrine (NE) cells show many similarities with the benign prostate. The NE cells in the benign prostate as like adenocarcinoma do not express luminal differentiation markers AR and PSA (Bonkhoff, 2001; Huang et al., 2006). Small cell

(neuroendocrine) carcinoma (SCNC) of the prostate are very rare and are mostly reoccurred in patients who had received hormonal therapy following conventional prostatic adenocarcinomas (Miyoshi et al., 2001). The tumor cells in SCNC also lack the expression of AR and PSA (Wagner and Huang, 2009) and are non-responsive to hormonal therapy (Papandreou et al., 2002). The SCNC is very lethal in case of distant metastasis leading to the patient's death within months of diagnosis (Tetu et al., 1987). Hence, it must be differentiated from prostatic adenocarcinoma (Tia et al., 2011).

The PC3 and LNCaP are the two commonly used cell lines for in-vitro studies. The PC3 cells have been derived from bone metastasis, whereas, the LNCaP cells are derived from the lymph node. It has been demonstrated that CD44 expression in PC cells increased its tumor initiation potential, which is not expressed in LNCaP cells (Palapattu et al., 2009; Marhaba et al., 2008; Patrawala et al., 2006; Tia et al., 2011).

It is also well known that PC3 cells are androgen independent (castration-resistant tumors) and do not express AR or PSA; on the other hand, LNCaP cells express these genes and are androgen dependent. Androgen withdrawal inhibits the growth of LNCaP cells as well as human adenocarcinoma. Prostatic adenocarcinoma, which comprises over 90% of all PCs, nearly always expresses AR and PSA irrespective of Gleason grade, so do the vast majority of castration-resistant PCs (Holzbeierlein, et al., 2004; Buchanan et al., 2001; Sadi and Barrack, 1993; Pertschuk et al., 1995; Culig et al., 1998). The LNCaP cells represent adenocarcinoma of the prostate (less aggressive) and PC3 cells (more aggressive) shows properties of prostatic SCNCs. It is very important for understanding their differences, mainly: (a) The LNCaP xenograft tumors are slow growing and less invasive, (b) The PC3 xenograft tumors are fast growing and more invasive, and (c) In in-vitro studies, PC3 cells are more migratory than LNCaP cells (Tia et al., 2011).

In treating the late stage of PC (castration-resistant PCs), AR is still expressed and is responsible for the survival of tumor cells (Sharifi, 2010). Hence AR and PSA negative cell lines are misleading and are not the proper representative of an aggressive form of castration-resistant PCs (Tia et al., 2011).

In most of the in-vitro studies, PC3 cells are considered as a representative of the aggressive form of advanced (castration-independent) stage of prostate adenocarcinoma. But, PC3 are more representative of prostatic SCNC (NE markers+, castration-resistant, CD44+, focal positivity for CK8) and LNCaP cells of adenocarcinoma (AR+, PSA+, androgen-dependent, diffuse positivity for CK8) (Tai et al., 2011). Hence, these two cell lines provide an excellent model for understanding molecular mechanisms as well as for developing novel therapies for prostate cancers.

1.7 Bacteriophages

Bacteriophages are the viruses that infect bacterial species. They are mainly classified as lytic and lysogenic phages based on their life cycles. Some bacteriophages like filamentous and/or pseudo lysogens have a complex life cycles which are yet to be understood thoroughly (Sanmukh et al., 2012). Bacteriophages have been used for lots of biomedical applications such as phage therapy against antibiotic resistant bacteria (Khairnar et al., 2013), development of novel phage differentiating methods through comet assay (Khairnar et al., 2014), etc.

Considering the inert nature of bacteriophages, they are also explored as a tool for cancer research and treatment (Sanmukh et al., 2017; Sanmukh and Felisbino, 2017). It has been reported that phages have peptides demonstrating anti-migratory and anti-proliferative activities naturally (Dabrowska et al., 2004a; Dabrowska et al., 2004b; Dabrowska et al., 2009). Also, the in-vitro and in-vivo interaction between mammalian cells and phage peptides with integrins via

RGD peptide sequence have been reported by various researchers; which can regulate adhesion properties of cancer cells, impairing their growth and proliferation (Ruoslahti and Pierschbacher, 1987; Pasqualini and Ruoslahti et al., 1996; Pasqualini et al., 1999; Rivinoja and Laakkonen, 2011; Dabrowska et al., 2004a; Dabrowska et al., 2004b).

Due to the importance of bacteriophages and their peptides as reported above, we selected three different phages for this in-vitro interaction studies between PC3 and LNCaP cell lines; namely, bacteriophage T4, bacteriophage M13 and bacteriophage MS2.

The bacteriophage T4 (Enterobacteria phage T4) infects *Escherichia coli*. It is a member of the T-even phages and can undergo only a lytic lifecycle and does not have the lysogenic lifecycle. It is a large phage in size (approximately 90 nm wide and 200 nm long). Its genetic material is DNA present in icosahedral head (capsid). It transfers its genetic material through tail for infecting its bacterial host. Through tail fibers phages detect its host (Ackermann and Krisch, 1997).

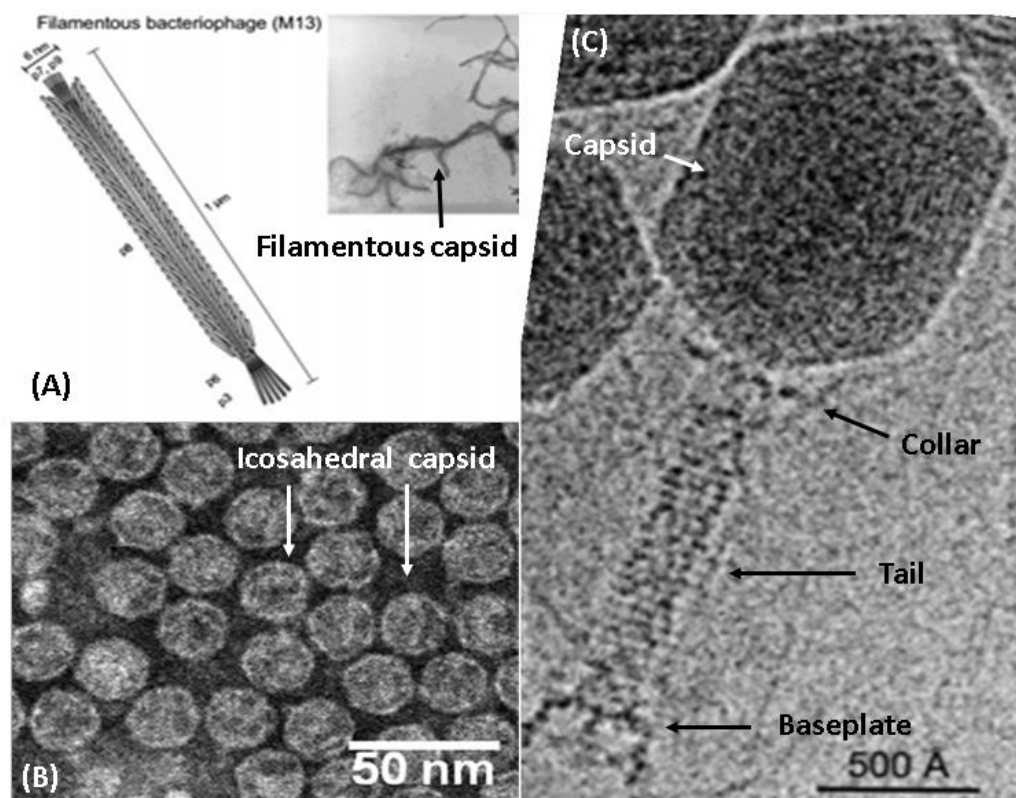


Figure 2. Transmission electron microscopy image of multiple M13 bacteriophages (A) (adaptation from Lee et al., 2013); The Cryo-TEM image of normal MS2 bacteriophage (B) (adaptation from Nguyen et al., 2011); and The Cryo-EM micrograph of phage T4 (C) (adaptation from Rossman et al., 2004)

The bacteriophage M13 is a filamentous phage and infects bacterium *Escherichia coli*. It has a single-stranded circular DNA. The coat of M13 phages is about 900 nm long and flexible, with the single-stranded genome (Speckthrie et al., 1992). The phage M13 life cycle is neither lytic nor lysogenic in nature. It multiplies within its host using all its cellular machinery without lysis and the infected cell remains normal except some effects like slow growth (Sanmukh et al., 2012). Due to this unique nature and properties exhibited by phage M13 they are used for many applications like recombinant DNA processes and nanotechnology. Similarly, phage MS2 is also an icosahedral phage particle. As compared with the T4 phage and M13 phage it is an RNA phage, it has a positive-sense single-stranded RNA as its genetic material. It is reported to infect the members of Enterobacteriaceae (Van Duin and Tsareva, 2006). It was first isolated and recognized as an RNA phage by Alvin John Clark followed by its complete sequencing in 1976 (Fiers et al., 1976). The first gene to be sequenced in MS2 phage was its coat protein. It was sequenced in 1972 by Walter Fiers and his team followed by complete sequencing of MS2 phage genes in 1976 (Min Jou et al., 1972; Erickson and Altman, 1979). The MS2 phage is around 27 nm in diameter with 180 copies of coat proteins within icosahedral head which encloses RNA (Strauss and Sinsheimer, 1963; Valegård et al., 1994). The MS2 phages have various applications from detection of RNA in living cells by MS2 tagging to tumor imaging and drug delivery applications (Glasgow and Tullman-Ercek, 2014).

1.8 Bacteriophages in Cancer Past, Present, and Future

The complete and up-to-date literature review for the role of bacteriophages in cancer research has been published as a review article in **Clinics in Oncology** and is included in the Annexure I. A book chapter with respect to non-specific interactions of phages with bacterial cells has been published with **De Gruyter, Germany** in a book entitled **Microbial Applications Recent Advancements and Future Developments** and is included in the Annexure II. Also, a short communication related to our preliminary findings related to bacteriophage as natural nanoparticles is reported in **Virology Research Journal** (Annexure III) and editorial promoting bacteriophages as an option for internal medicines is published in **Archives of General Internal Medicine** (Annexure IV).

2. JUSTIFICATION

The use of bacteriophages as therapeutic agents in the treatment of various types of cancers, including prostate cancer, either directly or as carriers of antitumor molecules, has been widely explored today. Thus, we believe that it is extremely relevant to evaluate the possible gene expression changes in two important prostate cancer cell lines namely, PC3 and LNCaP induced by the contact with bacteriophages T4, M13 and MS2. Previous studies have shown that bacteriophages can interact with mammalian cell membrane proteins, including integrins that recognize RGD sequences in viral proteins, as well as with fibronectin and other extracellular matrix molecules. Our approach using 2 cell lines becomes more appropriate considering that these cells represent a tumor responsive to androgenic stimuli (LNCaP) and another that is not responsive to androgens (PC3), and express different classes of proteins in their membranes. Also, our work can further validate the pros and cons with respect to the use of bacteriophages as a Nano-biomedicine against cancer.

3. OBJECTIVES

3.1 General Objectives

The aim of this study is to investigate how the natural bacteriophages interact with the cancer cell lines in-vitro through gene expression studies related to cell growth, migration, and proliferation.

3.2 Specific Objectives

- 1) To evaluate the binding of bacteriophages T4 and M13 with the PC3 cell line, to study the effect of bacteriophage T4 and M13 on different cancer progression genes related to cell growth and migration in PC3 cell lines.
- 2) To evaluate the effects of bacteriophage T4, M13 and MS2 on different cancer progression genes related to cell growth, proliferation, and migration in LNCaP cell lines.
- 3) To investigate whether the LNCaP cell line can be used for studying migration assay through our homemade pipette tip gap closure migration assay or Semi-Adherent Relative Upsurge method (s-ARU method).

4. RESULTS AND DISCUSSION

The results obtained from this research project are presented in the form of following 3 chapters. Two of which are research articles and one is a methodology article.

4.1 Chapter 1: Bacteriophage T4 And M13 Interactions with Human Prostate Cancer Cells (PC-3) alters Gene Expression and Impairs Cell Migration.

(Submitted to the Journal of Biochemical and Biophysical Research Communications)

4.2 Chapter 2: Characterizing interaction between LNCaP cell line with bacteriophages T4, M13 and MS2 for studying their effect on cancer cell survival.

(Submitted to the Journal of Clinical Virology)

4.3 Chapter 3: Development of pipette tip gap closure migration assay (s-ARU method) for studying migration assay in semi-adherent cell lines.

(Published in Cytotechnology)

4.1 CHAPTER 1:**BACTERIOPHAGE T4 AND M13 INTERACTIONS WITH HUMAN PROSTATE CANCER CELLS (PC-3) ALTERS GENE EXPRESSION AND IMPAIRS CELL MIGRATION**

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Running title: Bacteriophages alters gene expressions and impairs migration in the PC3 cells

Submitted to the Journal of Journal of Biochemical and Biophysical Research Communications

List of Abbreviations

ATCC: American Type Culture Collection

ECM: Extracellular Matrix

FBS: Fetal Bovine Serum

Hsp27: Heat shock protein 27

Hsp90: Heat shock protein 90

PBS: Phosphate buffered saline

PC3: Prostate cancer cell line

PFU: Plaque forming unit

PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase

PKB: Protein kinase B (also known as AKT)

Abstract

The non-specific interaction between bacteriophages and integrins have been reported in different cancer cells. Here we are trying to understand bacteriophage interactions with the PC3 prostate cancer cell line in-vitro for their possible role in gene alterations and application capabilities in developing novel cancer therapies. We are here with reporting the non-specific interaction of T4 and M13 bacteriophages with human PC-3 cells in-vitro and their possible effects on the expression of important genes including integrins, HSP27, HSP90 and androgen receptor (AR). The PC-3 tumor cells (80% confluent) were exposed to 1×10^7 pfu/well concentration of lytic bacteriophage T4 and filamentous bacteriophage M13 separately, along with vehicle cells without phages as a control. After 24 hours of exposure, the cells were processed for histochemistry, scanning electron microscopy, wound-healing migration assay and gene expression by Real-Time quantitative PCR (qPCR). The qPCR was performed for analyzing the gene expression profile of integrin ITGAV, ITGA5, ITGB1, ITGB3, ITGB5, HSP 27, HSP 90 and AR. Our studies revealed that PC3 cell line interacts with bacteriophage T4 and M13 as observed from histochemistry and scanning electron microscopic studies. It was also confirmed through the significant increase in gene expression changes of ITGAV, ITGA5, ITGB1, ITGB3, ITGB5 and a marked decline in HSP27, HSP90, and AR gene expressions within these cells after phage exposure. PC3 cells also exhibited reduced migration activity when exposed to T4 and M13 phages, mainly M13. Our results suggest that the natural bacteriophages can interact with cell surface receptors increasing their expression and cell adhesion properties. The down-regulated gene expressions of HSP27, HSP90, and AR suggest that phages can naturally modulate crucial cancer genes and have potential in near future for anti-cancer therapies.

Keywords: bacteriophage, prostate cancer, integrin, PC3, nanoparticle, cellular migration

Introduction

Prostate cancer (PCa) is the second most diagnosed cancer in the American continent [1]. In most cases, due to its early detection and effective treatment it has a good outcome [2,3]. But, as in most cancers, advanced stages have various limitations, which restrict proper implementation of treatments and leads to the emergence of personalized medicines [4]. Considering the androgen deprivation therapy for prostate cancer, which mainly leads to the castration-resistant or androgen-independent prostate cancer, it seems that there is a need for alternative effective targeted therapy [5]. Due to the increasing orientation for various nanotechnological approaches for cancer cell targeting, detection, treatment and cancer cell killing; searching effective nano-material for nanoparticle remains a big challenge [5, 6, 7]. Along with the nanoparticles investigated recently, Bacteriophage, a naturally available nanoparticle, has been effectively utilized for its anti-microbial applications for different microbial pathogens and have been efficiently utilized for various application purposes encompassing different fields including medicine [8, 9,10].

It is already reported that bacteriophages are present in our body [11] and has a significant influence on the immune system naturally [12,13,14]. The use of bacteriophages for targeting cancer cells in vitro and in vivo has emerged as a promising tool since Kantoch and Mordarski demonstrated phage binding to cancer cells [15, 16, 17, 18].

Currently, through in-vivo phage display, it is demonstrated that the molecular heterogeneity of the vascular endothelium, as well as organ targeting, is possible through phage peptides [19, 20, 21] and mapping of human vasculature has been carried out through phage display [22, 23], which is having a huge impact on current cancer research and treatment [24, 25]. Also, modified bacteriophages, such as engineered phages, hybrid phages, etc., are

considered to be of tremendous importance from cancer cell targeting to drug delivery as reported by various researchers [26, 27, 28, 29].

Here, we report the interaction studies between two different types of bacteriophages with human PC-3 cells, to understand its non-specific binding and possible effects on the cell morphology, migration along with gene expression alterations for integrins, HSP27, HSP90, and AR.

Materials and Methods

PC3 cancer cell line culturing

The experiment was designed with PC-3 cell line obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). The PC3 cells were cultured initially in 25 cm² culture flask (Qiagen, Crawley, UK) with RPMI medium supplemented with 10% fetal bovine serum (SFB) (Invitrogen™) and 1% antibiotic/antimycotic solution (Invitrogen™) and were kept under strict sterile conditions and inside a 5% CO₂ incubator (Thermo Scientific™). The cells were incubated for 24 hours at 37⁰C. They were later expanded to 75 cm² culture flask (Qiagen, Crawley, UK). For experimental purpose, PC3 cells were seeded at 2 X 10⁴ cells/cm² per well in 6-well culture plates. All the experiments and analysis were carried out as per the standard procedures as well as guidelines provided by respective authorities.

PC3 Cell exposures to Bacteriophages T4 and M13

The bacteriophage M13 (New England Biolab's Inc, Ipswich, USA) and T4 (Carolina Inc, Burlington, UK) were purchased. The dilutions of each phage were standardized at 1 X 10⁷

pfu/ml for interaction studies. The concentration of each phage for every experiment was 10^7 pfu per well of 6-well plate. Upon reaching 80% of confluence, PC3 cells were treated for 24 hours with bacteriophages M13 and T4 (10^7 pfu/well concentration), separately, in 6-well culture plates. Each experiment was carried out in triplicate with control of PC3 vehicle (PBS) cells without phage. Others replicates were carried out in 6-well plates, separately for protein extraction, and in 12-wells plates containing cover-slips in the bottom for histochemistry and scanning electron microscope studies.

Histochemistry studies

The histochemistry study for understanding the morphological changes associated with the PC3 cell interactions with phage T4 and M13 was carried out by using Eosin-Methylene blue staining for the cells fixed with Phage treated and untreated PC3 cells followed by light microscopic (Leica) observations.

Extraction of RNA and cDNA synthesis for qPCR studies from PC3 cells

The RNA extraction was carried out by using All Prep DNA/RNA/Protein extraction kit Follow the Manufacturer's Instructions (Qiagen, Crawley, UK). The RNA was quantified using a NanoVue™ Plus spectrophotometer (GE Healthcare, USA), which also allowed an estimation of RNA purity by measuring the absorbance at 260 nm (RNA quantity) and 280 nm (protein quantity). Only samples with 260/280 ratio ≤ 1.8 were used. RNA reverse transcription was performed using a High Capacity cDNA Archive Kit (ThermoFisher Scientific, USA) according to the manufacturer's guidelines.

The mRNA expression levels were measured by qPCR using QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, USA). All qPCRs performed were compliant with the Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines. The cDNA samples were amplified using SYBR® Green Master Mix (ThermoFisher Scientific, USA) and specific primers were synthesized by Invitrogen (USA). The reactions were performed at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The specificity of each primer set was evaluated by the dissociation curve at the end of each PCR reaction, which confirmed the presence of a single fluorescence peak. The relative quantification of expression was performed by the $2^{-\Delta\Delta C_t}$ method [30] using the DataAssist™ v3.01 software (Thermo Fisher Scientific, USA).

The reactions were performed in triplicate for 16 target gene procured from New England Biosciences viz, ITGA5, ITGAV, ITGB1, ITGB3, ITGB5, HSP90, HSP27, AR, along with endogenous controls ACTB. According to the expression stability among all samples, the reference gene ACTB was used to normalize mRNA expression.

Wound Healing Assay

The PC3 cell migration studies were carried out in 12 well plates with and without treatment of bacteriophage T4 and M13. The PC3 cells were cultivated in 12 well plate at 4×10^4 cells/cm² concentration until 100% confluence was achieved. To perform this, assay a monolayer was scratched across the well with a 100-200 μ l pipette tip, using a sterile 6-well culture plate lid as a support. After making monolayers, each well of the 12 well plate was then washed twice with D-PBS (GIBCO/Invitrogen™) to remove cell debris and were added with fresh RPMI media. Each three set of monolayers were later labeled as control, phage T4 treated

and phage M13 treated for experimental purpose. The bacteriophage T4 and M13 were taken used in equal concentration i.e. 1×10^7 pfu/ μ l. For phage T4 and M13 treatment, each of three wells was inoculated with 10 μ l of 1×10^7 pfu/ μ l of phage culture medium separately. The "zero" hour reading was recorded for each control and treatment using an inverted phase contrast microscope with the digital camera followed by 12, 24 and 48 hours reading.

Scanning electron microscopic studies

The scanning electron microscope was carried out for the PC3 cells grown over coverslips treated or untreated with T4 and M13 phages, at a final concentration of 1×10^7 pfu/ μ l. After attaining 80% of confluence, cells were treated or not for 24 hours. After phages exposure, the cells were fixed with 2.5% glutaraldehyde in phosphate buffer pH 7.2 (0.2 M). After fixation with glutaraldehyde, the pellets are washed in PBS and fixed in osmium tetroxide 1% in PBS, washed in PBS, dried in CO₂ critical point and coated with gold particles, according to the protocols of Centre of Electron Microscopy – IBB (UNESP). Cells were observed under scanning electronic microscope Quanta 200 (FEI Company, Hillsboro, Oregon, USA) operating at 80 kV.

Statistical analysis

Statistical analyses were performed using GraphPad Prism® software (version 5.00, Graph Pad, Inc., San Diego, CA). The results were submitted to analysis of variance (ANOVA), followed by the Tukey–Kramer test, and the data were expressed as the mean \pm SD. Differences were considered statistically significant when $p < .05$.

Results

Morphological study

The phage T4 and phage M13 treated PC3 cells appeared more fusiform as compared with the untreated PC3 cells, whereas no changes in mitotic behavior were observed (Figure.1). Using a scanning electron microscope, it was observed that bacteriophages T4 and M13 were attached to the cell surface (Figure 2).

Wound Healing Assay

The interaction of PC3 cell line with the bacteriophage T4 and M13 was also evaluated by scratch wound healing assay. It was observed that bacteriophage M13 was more effective in migration inhibition as compared with the phage T4 (Figure 3).

Effect of T4 and M13 exposure on PC3 cells gene expression

Bacteriophages T4 and M13 exposures induced a significant increase in mRNAs expressions related to integrin genes after 24 hours. The relative mRNAs expressions for ITGAV, ITGA5, ITGB3, and ITGB5 were significantly increased, whereas, those for HSP90 and AR were significantly decreased (Figure 4). However, HSP27 gene expression was significantly reduced only after phage M13 exposure and ITGB1 was unchanged with both the treatments.

Discussion

Bacteriophages represent an important tool for exploring tumor cell nanoparticles interactions and drug delivery. The results presented in this work clearly have shown the possible interaction between the PC3 cells with the natural bacteriophages T4 and M13, consistent with previous studies [14, 31, 32, 33].

Bacteriophages represent an important tool for exploring tumor cell nanoparticles interactions and drug delivery. The results presented in this work clearly have shown the possible interaction between the PC3 cells with the natural bacteriophages T4 and M13, consistent with previous studies [14, 31, 32, 33].

Our findings strongly have shown up-regulation of several class of integrins in PC3 cells which could be a direct or an indirect effect of the phage peptides with integrins via RGD peptide sequence [34, 35, 36, 37, 38]. This interaction and integrin up-regulation may promote more adhesion properties of cancer cells, impairing their growth and proliferation [34, 37, 13, 14]. Studies have shown that integrin binding and up-regulation can act as a positive or negative factor for cell migration and proliferation [34, 37]. Our migration assay results suggest that this interaction reduces cell proliferation and migration as observed in melanoma cells [13,14].

Another interesting result of our work was that phage interaction reduces the expression of HSP90 [31, 39, 40]. HSP90 is a crucial chaperone heat shock protein with several targeting proteins in the cytoplasm, involved in several processes of the cells including cell proliferation, stress conditions and steroid hormones activation, such as AR [41, 42, 43]. It is also important to mention that androgen receptor expression was also down-regulated by phage exposures [31]. The suppression activity of HSP90 has been widely explored in cancer treatment, including castration-resistant prostate cancer [44, 45, 42, 46]. In this sense, phage down-regulation of HSP90 and AR in prostate cancer cells seems to be a promising approach for the combined treatment of advanced prostate cancer that doesn't respond to canonical anti-androgenic treatment.

In conclusion, natural bacteriophages interact with the prostate cancer cell, which can stimulate gene expression alterations. These effects on the specific gene expression reveal that phages have broad range binding properties through their proteinaceous external coat, which may be considered as a natural peptide display for inducing this effect and can be significantly useful for anti-cancer therapies.

Disclosure Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' contributions

SGS and SLF conducted most of the experiments and analysis and wrote the manuscript. SGS, NJ, CNB, SAAS, and BOSD generated data and performed analyses or both. LAJJ, FKD, and SLF supervised the project. SGS and SLF were responsible for overseeing the manuscript. All authors discussed the results and reviewed the manuscript.

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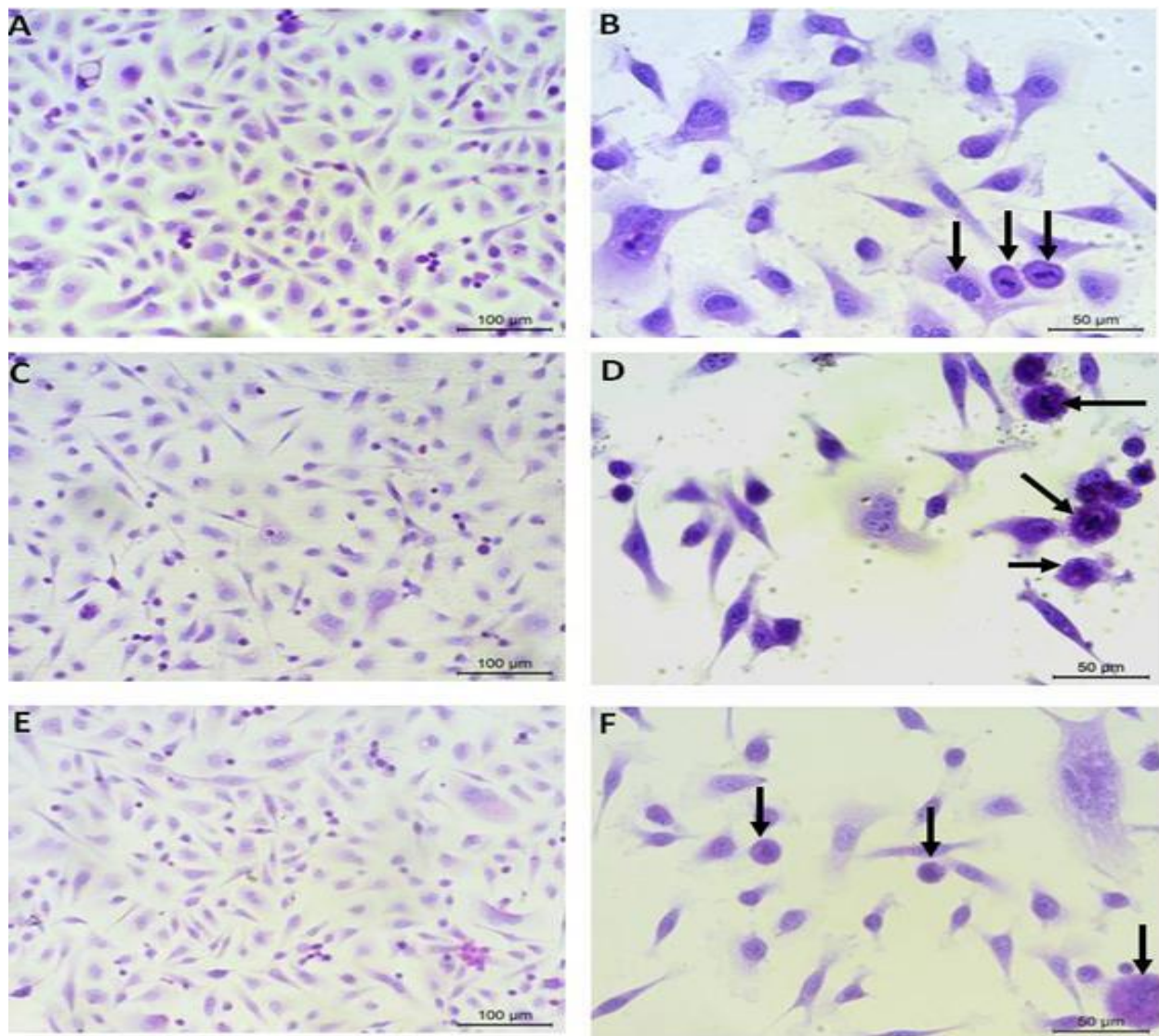


Figure 1. Prostate tumor cells PC3 stained with Hematoxylin and eosin. Untreated PC3 cells (A and B); PC3 cells exposed to the bacteriophage M13 (C and D); PC3 cells exposed to the bacteriophage T4 (E and F). Note the difference in morphology, which appeared more fusiform in treated cells. The arrows point to mitotic figures, with no changes in its quantity, apparently. Scale bars: A, C and E = 100 µm; B, D and F = 50 µm.

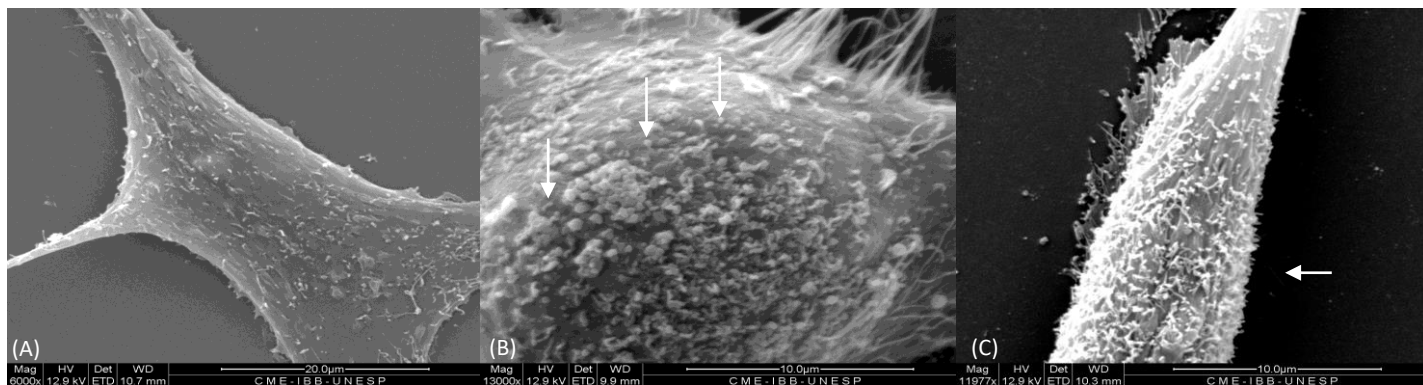


Figure 2. The scanning electron microscopic images were obtained from the bacteriophage treated and untreated PC3 cells. The control (A), bacteriophage T₄ treated (B), & bacteriophage M13 treated (C) PC3 cells shows that phages interact with the cell surface, which is responsible for alteration in gene expression significantly.

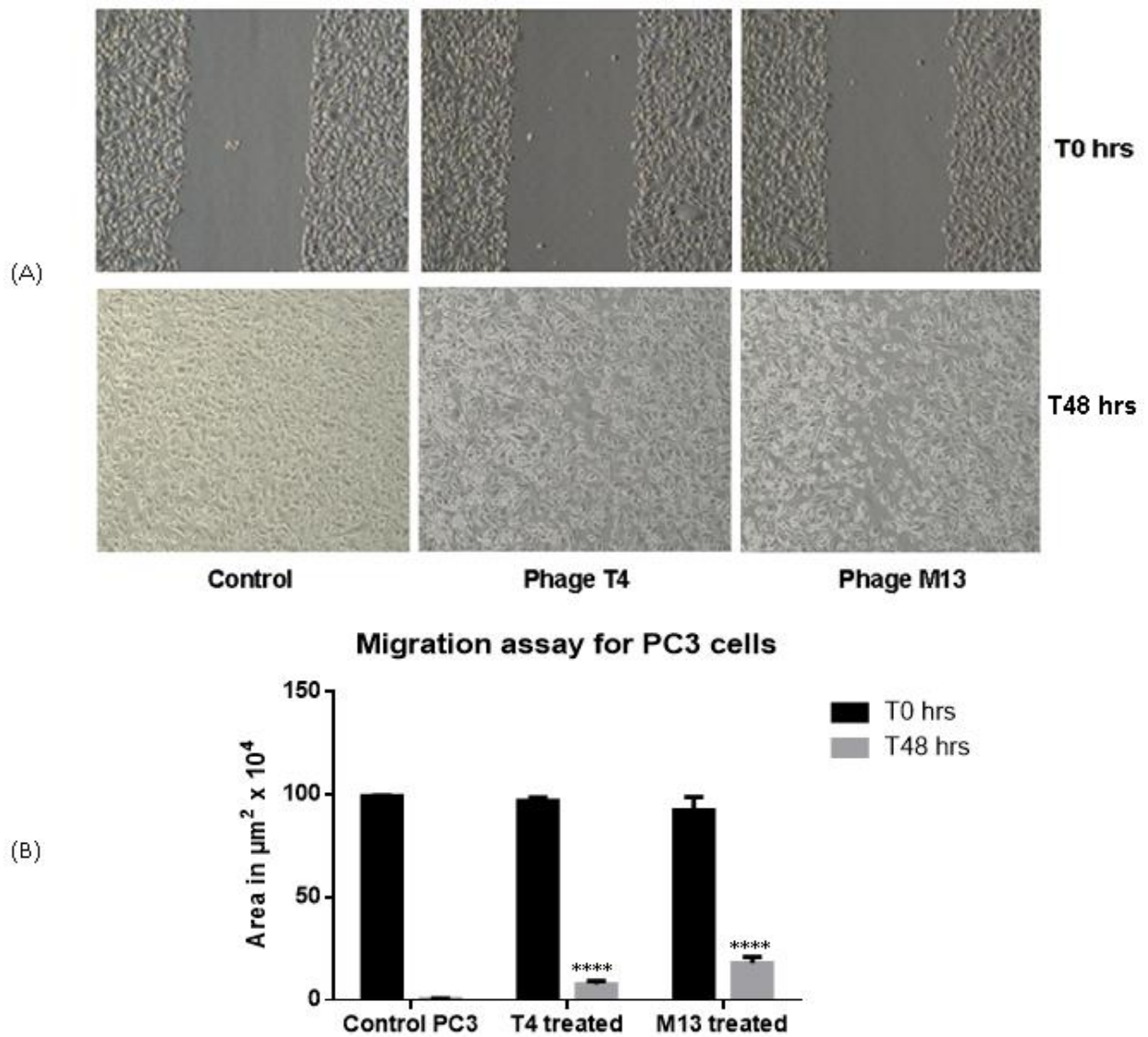


Figure 3. The scratch assay performed for PC3 cell line clearly shows that PC3 cells migration is affected by both the phage T4 and M13. It can be confirmed that in the 48 hours' time study phage M13 slow down the migration significantly as compared with the phage T4 (A). Statistical analysis of wound healing assay for bacteriophage T4 and M13 treated PC3 cells against control PC3 cells (where control represents 100% of migration from the average of three samples sets). (P value < 0,0001)

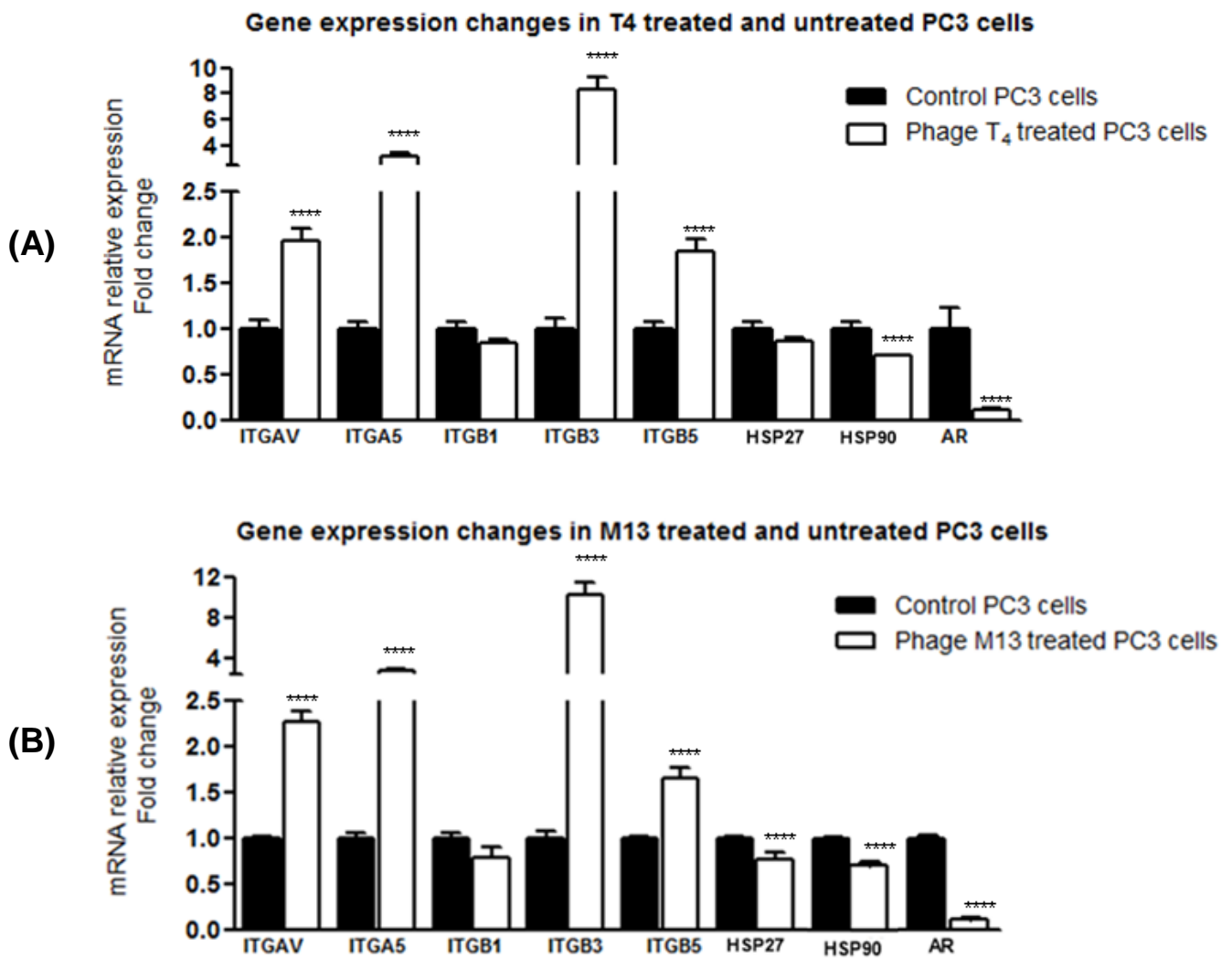


Figure 4. The histogram representation of effect on PC3 cells gene expression after interaction with bacteriophage T₄ (A) and bacteriophage M13 (B). Genes which were overexpressed significantly by both phages include integrins (ITGAV, ITGA5, ITGB3 & ITGB5) whereas androgen receptor (AR) and HSP90 were significantly suppressed in both phages and HSP27 was significantly suppressed only with phage M13. Expressions were normalized by ACTB expression. (P value <0.001)

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4.2 CHAPTER 2:**CHARACTERIZING INTERACTION BETWEEN LNCAP CELL LINE WITH BACTERIOPHAGES T4, M13 AND MS2 FOR STUDYING THEIR EFFECT ON CANCER CELL SURVIVAL**

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List of Abbreviations

ATCC: American Type Culture Collection

ECM: Extracellular Matrix

FCS: Fetal Calf Serum

HSP27: Heat shock protein 27

HSP90: Heat shock protein 90

LNCaP: Lymph Node Carcinoma of the Prostate

PBS: Phosphate buffered saline

PFU: Plaque forming unit

PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase

PKB: Protein kinase B (also known as AKT)

PGC1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

ERR α : Estrogen Related Receptor alpha

s-ARU method: Semi-Adherent relative upsurge method

PKCepsilon: Protein kinase C-epsilon

PKD3 pathway: Protein kinase D3

hCGbeta: Human Chorionic Gonadotropin beta

Abstract

Background: The LNCaP cell line have been used to investigate possible effects of bacteriophages on cell signalling pathways important for the growth and progression of PCa.

Objectives: To characterize the interaction of bacteriophage T4, M13, and MS2 with the LNCaP prostatic epithelial cell line to evaluate their role in modulating different genes related to cancer cell survival.

Study-design: The LNCaP cells were exposed to 1×10^7 pfu/well concentration of bacteriophage T4, M13, and MS2 separately, along with vehicle cells without phages as a control. After 24 hours of exposure, the cells were processed for histochemistry, western blotting for AKT, PI3K, Phospho-AKT and Phospho-PI3K activation, and for gene expression analysis of integrins (*ITGAV*, *ITGA5*, *ITGB1*, *ITGB3*, *ITGB5*), *HSP27*, *HSP90*, *AKT*, *PI3K*, *MAPK1*, *MAPK3*, *STAT3*, *PGC-1 α* , *PSA*, and *AR*.

Results: It was observed that LNCaP cell exposure to bacteriophage M13 significantly down-regulated most of the genes namely *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *MAPK1*, *MAPK3*, *HSP90*, *PSA* as compared to phage T4 and MS2, whereas it up-regulates *HSP27*, *PGC-1 α* , *STAT-3*, and *AR* genes. However, no activation of AKT, PI3K, phospho-AKT, and phospho-PI3K was observed in this cell after all three bacteriophages exposure.

Conclusions: Our results suggest that from bacteriophage T4, M13, and MS2, phage M13 interacts with the cell surface of LNCaP cells effectively and mediate gene expression changes with suppressive effects for cancer cell survival and growth. Bacteriophages, naturally or modified, could work as a therapeutic approach to prostate cancer.

Keywords prostate cancer, gene expression, integrin, bacteriophage, nanoparticle

1. Background

Prostate Cancer (PCa) is the second most leading cause of deaths in men globally [1, 2]. The PCa is curable in most of the cases if detected earlier in a person before causing distant metastasis and spreading to the bone and other parts of the body [3, 4]. In the case of PCa, besides the importance of earlier detection and diagnosis, it has become imperative new options of treatment for advanced stages which are resistant to anti-androgenic therapies and chemotherapies [5, 6].

Several reports have been published with respect to effects of bacteriophages on cancer cells in-vitro as well as in-vivo, which are very promising [7, 8, 9, 10]. Also, hybrid phages or modified phages have been developed by various groups worldwide to detect, target and attack specific cancer cells for diagnosis of different cancers including PCa [10, 11, 12, 13]. Nevertheless, very few reports have been published related to the effect of natural phages on cancer cell lines in-vitro for understanding gene expression changes associated with cancer cell survival.

Here, our objective was to report important findings associated with the direct interaction of bacteriophage T4, M13, and MS2 with LNCaP prostate cancer cell line. We have targeted specific genes important for cancer cell growth, proliferation, migration, and invasion. Also, it is very essential to know how natural phages affect cancer cells since they are present normally in our body [7, 8, 14, 15]. In this sense, it appears mandatory to evaluate their potential as nano-particles with anti-cancerous activity.

2. Materials and Methods

2.1. LNCaP cell culture and staining

LNCaP cells were purchased from American Type Cell Culture (Manassas, Virginia, USA). The LNCaP cells were cultured initially in 25 cm² culture flask (Qiagen, Crawley, UK) with RPMI 1640 medium (GIBCO / Invitrogen™) supplemented with fetal calf serum (FCS) (Gibco / Invitrogen™) 10% 50 µg / ml penicillin, 50 µg / ml streptomycin and 0.5 µg / ml amphotericin B (Gibco / Invitrogen™) and were incubated in a CO₂ incubator for 24 hours at 37⁰C. They were later expanded in 75 cm² culture flask (Qiagen, Crawley, UK) for interaction studies and seeded at 2 X 10⁴ cells/cm² in each well of 6-well culture plates. All the experiments and analysis were carried out as per the standard procedures and provided guidelines. The medium was changed every 2 days followed by daily monitoring of the cells in the inverted microscope (Zeiss Axiovert®). After 80% confluency, cells were subjected to 0.05% trypsin (Gibco / Invitrogen™) and transferred to new culture plates or to 6 well plates (Millipore Corp. or Corning™) to initiate the different treatments.

2.2. LNCaP cell exposure to Bacteriophage T4, M13 and MS2

The bacteriophage M13 (New England Biolab's Inc, Ipswich, USA), T4 (Carolina Inc, Burlington, UK) and MS2 (Zeptomatrix Corporation Inc., NY, USA) were purchased. Upon reaching 80% of confluence, LNCaP cells were treated for 24 hours with bacteriophages T4, M13, and MS2 (10⁷ pfu/well concentration), separately, in 6-well culture plates. Each experiment was carried out in triplicate with control of LNCaP cells treated with vehicle (PBS) without phage. All replicates were carried out in 6-well plates, separately for protein extraction and total RNAs extraction, and in 12-wells plates containing cover-slips in the bottom for histochemistry.

2.3. Histochemistry studies

The LNCaP cells were exposed to bacteriophage T4, M13, and MS2 treatment for 24 hours and were washed in PBS and fixed with 10% formaldehyde in PBS for 30 minutes. The cells were washed in PBS and stained by Hematoxylin-eosin. The cover-slips were dried in ethanol and mounted in a glass slide with Permount[®] and observed in a Leica DMLB photonic conventional microscope (Leica Inc.).

2.4. Extraction of RNA and cDNA synthesis for qPCR studies in LNCaP cells

For total RNA extraction, the culture medium was aspirated, cells were washed with 1x PBS (and later aspirated). Total RNAs were extracted using the extraction kit All Prep DNA/RNA/Protein extraction kit (Qiagen, Crawley, UK) according to the instructions of the manufacturer. The total RNA extracted was quantified by using NanoVue (GE Healthcare, USA). Two micrograms of total RNAs were reverse transcribed using Kit high-capacity RNA-to-cDNA kit (Life Technologies, USA) in 20 µl reaction according to the instructions of the manufacturer.

For the Real-Time PCR, the Power SYBR Green / ROX qPCR Master Mix reagent (2x) (Applied Biosystems) was used. The total reaction volume per sample was 10 µL (5.0 µL Power SYBR Green, 0.8 µL of each forward and reverse oligonucleotide [800 nM], 3.2 µL nuclease-free water, 1 µL cDNA), performed in triplicate using 384 well plates. The reaction was performed in the QuantStudio 12k Flex thermal cycler (Applied Biosystems), the results were evaluated by QuantStudio 12k Flex Real-Time PCR System v1.1 program. The reaction consisted of the following cycling: step 1 at 50 ° C for 2 minutes and 95 ° C for 2 minutes; step 2 at 95 ° C for 1 second and step 3 at 60 ° C for 30 seconds; steps 2 and 3 were repeated 40 times; dissociation curve with incubation at 95 ° C for 15 seconds and at 60 ° C for 1 minute

with subsequent increase in temperature from 60 °C to 95 °C at the rate of 0.15 °C per second.

For the calculation of gene expression, the $\Delta\Delta C_t$ method was used [16], which is based on the exponential PCR reaction. For this the formula was used: $QR = 2^{-\Delta\Delta C_t}$, where QR represents the level of gene expression; C_t represents the amplification cycle in which each sample exhibits exponential amplification; ΔC_t refers to the difference between the C_t of the amplified sample for the target gene and the C_t of the same amplified sample for the reference gene, and $\Delta\Delta C_t$ represents the difference between the ΔC_t of the sample of interest at a given time and the ΔC_t of the reference sample.

The reactions were performed in triplicate for 15 target gene (Gibco / Invitrogen™), *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *HSP90*, *HSP27*, *AR*, *PSA*, *AKT*, *PI3K*, *MAPK1*, *MAPK3*, *PGC-1 α* and *STAT3* with 2 endogenous controls *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and Beta-Actin in Real-Time PCR System AB7900 (Applied Biosystems) according to the manufacturer's instructions. The values for all samples are normalized by the ratio obtained between the target gene and the reference gene *GAPDH* and Beta-actin.

2.5. Extraction of Proteins from LNCaP cells and Western Blotting

For protein extraction, the culture medium was aspirated, cells were washed with 1x PBS (and later aspirated). Protein extraction was obtained through RIPA buffer (Qiagen, Crawley, UK). About 30 μ g protein was diluted in the sample buffer, heated and separated on 10% SDS-PAGE electrophoresis on Mini-Protean III system (Bio-Rad, California, USA). Separated proteins were transferred to nitrocellulose membranes 0.45 micrometers (Bio-Rad Laboratories, Hercules, USA), and were blocked with 5% skimmed milk diluted in TBS-

Tween. After washing these membranes were incubated with primary antibodies against total AKT, total PI3K and β -actin (Santa Cruz Biotechnologies, Dallas, USA) with a dilution of 1:1000. After overnight incubation, they were washed in TBS-Tween and membranes are incubated with secondary antibody (peroxidase-labeled) (Santa Cruz Biotechnologies, Dallas, USA) and developed by the detection system using western blotting detection reagent (Santa Cruz Biotechnologies, Dallas, USA). The ECL signals were captured using a CCD camera (ImageQuant LAS 4000 mini®; GE Healthcare™). The integrated optical density (IOD) of the bands were quantified using the ImageJ™ software for each sample (as triplicates).

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism® software (version 5.00, Graph Pad, Inc., San Diego, CA). The results were submitted to analysis of variance (ANOVA), followed by the Tukey–Kramer test, and the data were expressed as the mean \pm SD. Differences were considered statistically significant when $p = < 0.05$.

3. Results

3.1. Histochemistry studies

The histochemistry studies of LNCaP cells exposed to bacteriophage T4, M13 and MS2 showed no significant morphological alterations (figure 1).

3.2. Phage exposure and PI3K/AKT pathway activation in LNCaP cells

The treatment of LNCaP cells with phages T4, M13 and MS2 did not induce significant changes in both total and phosphorylated forms of PI3K and AKT (Figure 2).

3.3. Effect on LNCaP cell gene expression after exposure to bacteriophages T4, M13, and MS2

Gene expression analysis of LNCaP cells after exposure to phages T4, M13, and MS2 compared with the untreated cells showed up-regulation for *AR*, *STAT-3* and *PGC-1 α* , whereas, those for *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *HSP90*, *MAPK-1*, *MAPK-3* were decreased significantly for phage M13 treated cells, except *ITGAV* for phage T4 and *ITGB1* for both phage T4 and MS2 (Figure 3). *HSP27* gene expression was slightly increased by M13 and MS2 treatment, while it was slightly reduced by phage T4 treatment.

4. Discussion

Considering the importance of phage peptides in regulating important genes within cancer cells and tumors reported for the last three decades [17, 18, 19, 20, 21]; its mandatory to investigate bacteriophages due to their natural availability in our body as well as their role in influencing the immune system [7, 8, 9, 22].

Our results clearly present the possible effect of phage T4, M13 and MS2 interaction with LNCaP cell line and interfering with the genes important for cancer cell progression. This can be interpreted from our findings which have shown considerable down-regulation of integrin genes in LNCaP cells along with *MAP* kinases, *HSP90* and *PSA*; Also, upregulation of *PGC-1 α* [23] has been reported to suppress prostate cancer growth and metastasis as it is downregulated in prostate cancer. *PGC-1 α* activates an estrogen-related receptor alpha

(ERR α)-dependent transcriptional program to elicit a catabolic state and metastasis suppression. It has been observed that the PGC1 α -ERR α pathway exhibited prognostic potential in prostate cancer, which can be used for prostate cancer stratification and treatment [23].

We also observed that LNCaP cells and bacteriophage interaction affected negatively cell migration as well as growth, using our homemade developed pipette tip gap closure migration assay (s-ARU method) [published data]. Phage exposure also increased gene expression of STAT3 and AR, but with no changes in PI3k and AKT signaling pathways, which suggests that this up-regulation is not inducing pro-survival or cell proliferation as well as PSA secretion, since, PSA were downregulated too [published data].

In our previous studies with PC3 cells, it was observed that HSP90, which possibly affects *RUNX2*, which is an osteogenic transcription factor, has a role in prostate cancer cell growth and migration [24, 25, 26]. Since, this complex act through α v β 3 integrin-Smad5-RUNX2-receptor activator of NF- κ B ligand (RANKL) signaling axis [25], the down-regulation of *HSP90* along with other integrins seems to affects its function and can cause suppression of prostate cancer cell migration as observed in previously reported works in case of PC3 and LNCaP cells [24, 25, 26, published data].

Another interesting result of our work is about *MAPK1* and *MAPK3* down-regulation by M13 and MS2 exposure. These two genes are very important in human prostate cancer cell growth [27, 28, 29]. *MAPK3* (*ERK1*) is associated with prostate cancer cell growth, survival and invasion through PKCepsilon/PKD3 pathway [27, 30]. *MAPK3* gets phosphorylase in response to *hCGbeta* (human chorionic gonadotropin beta) to promote prostate cancer cell migration and cell motility [28] and its activation through *PI3K* signaling helps in prostate cancer cell proliferation [27, 28, 29, 30]. So, our results suggest that phages exposures affect negatively cell proliferation and survival.

Taking together, we can consider bacteriophage as an effective and efficient nanoparticle as it is naturally available within our body and has no adverse immune response associated with it in-vivo and in-vitro. Our purpose for conducting this study was to demonstrate that phages being a natural nanoparticle are capable of influencing cancer cell growth, proliferation and migration through gene expression changes and can be used to develop modified phage-based therapeutic products for cancer treatment. These effects on the specific gene expression reveal that phages have broad range binding properties through their proteinaceous external coat, which can be implemented for designing novel cancer therapies effectively.

Disclosure Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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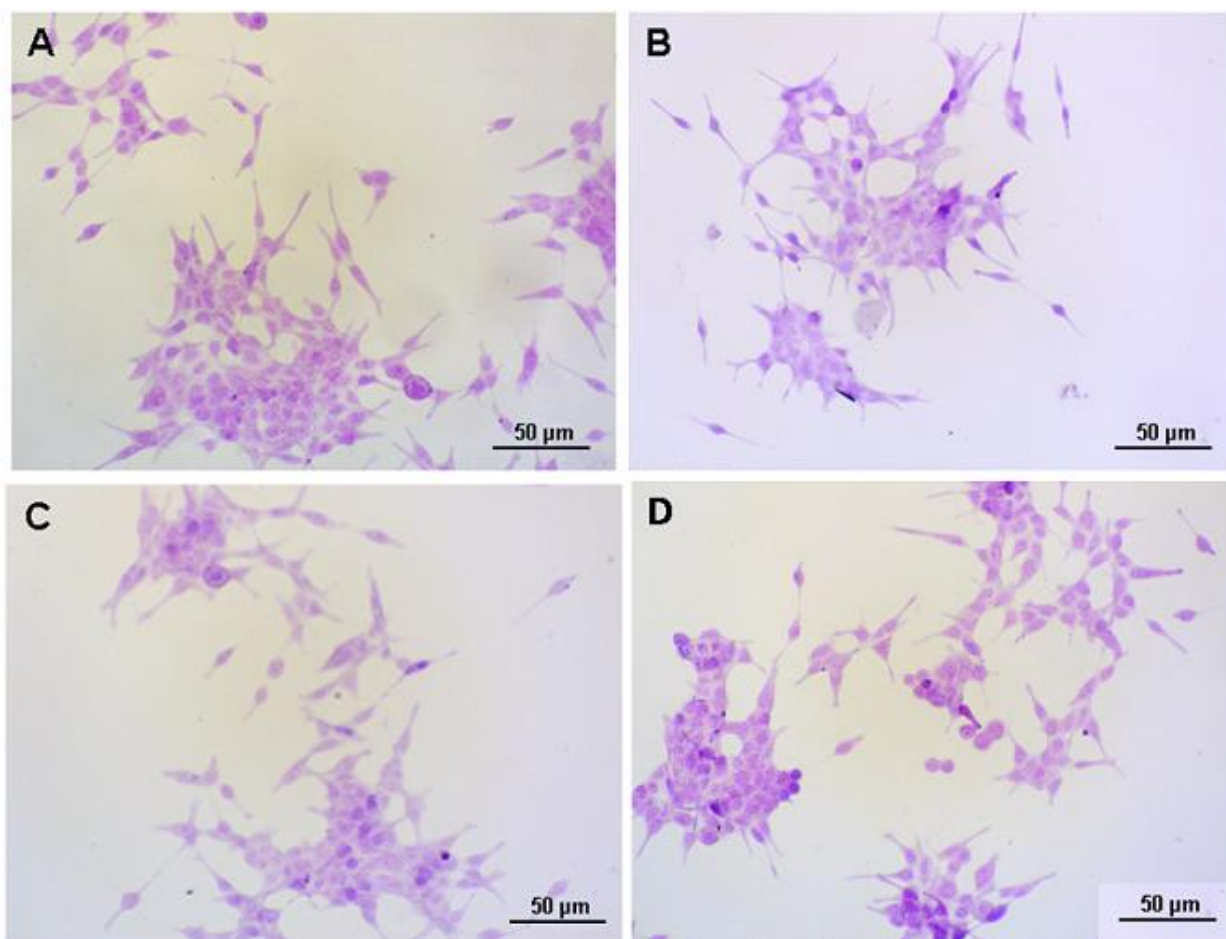


Figure 1. Representative images of LNCaP cells stained with Hematoxylin-eosin. A) untreated LNCaP cells. B) LNCaP cells exposed to the bacteriophage M13. C) LNCaP cells exposed to the bacteriophage MS2. D) LNCaP cells exposed to the bacteriophage T4. No significant morphological differences were observed among treated and untreated cells. Scale bars = 50 µm.

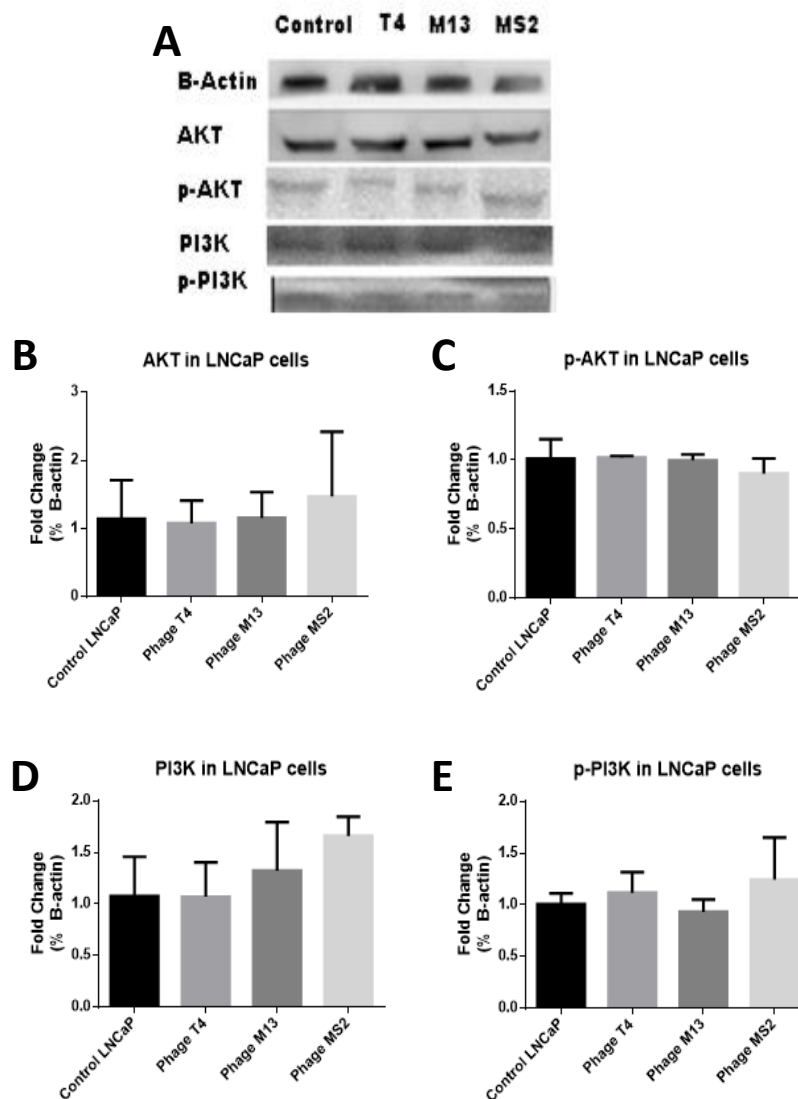


Figure 2. A) Representative images of Western Blotting results for protein expression of AKT, p-AKT, PI3K and p-PI3K by LNCaP treated and untreated (Control) by 1×10^7 bacteriophages T4, M13 and MS2 for 24 hours. B-D) Quantitative analysis of the bands of each protein analysed in the different experimental conditions. The data are expressed as mean \pm standard deviation of integrated optical density of the target protein bands after normalization by beta-actin protein expression. No significant differences among control and treated cells were observed.

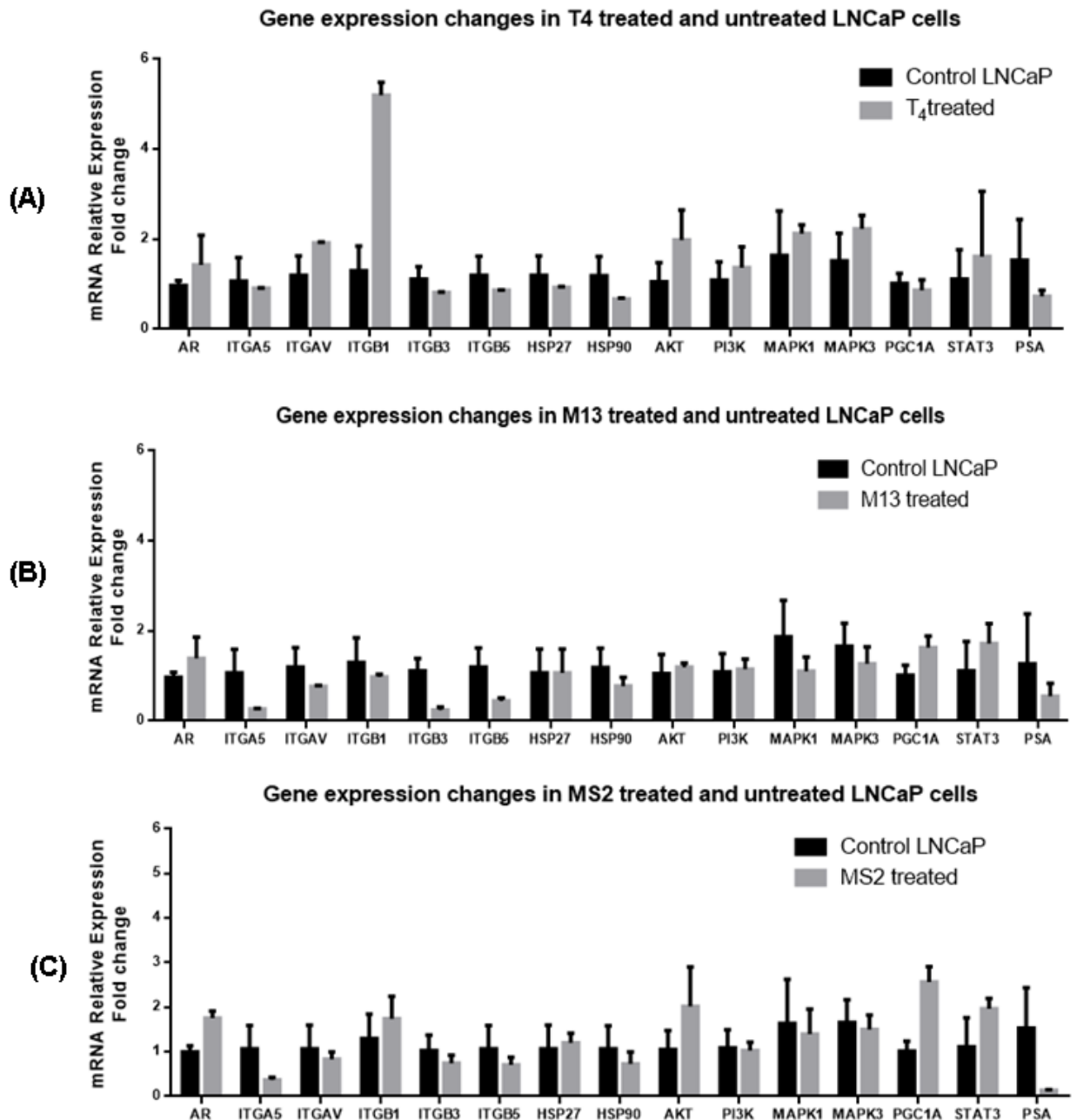


Figure 3. Histogram representation of effect on LNCaP cells gene expression after interaction with (A) bacteriophage T4, (B) bacteriophage M13, and (C) bacteriophage MS2. After T4 phage exposure *AR*, *ITGAV*, *ITGB1*, *AKT*, *PI3K*, *MAPK1*, *MAPK3* and *STAT3* were upregulated and *ITGA5*, *ITGB3*, *ITGB5*, *HSP27*, *HSP90*, *PGC1A* and *PSA* were down regulated. After M13 exposure *AR*, *PGC1A*, *STAT3* were upregulated; *ITGA5*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *HSP90*, *MAPK1*, *MAPK3*, *PSA* were down regulated whereas, no significant changes were observed in *HSP27*, *AKT* and *PI3K*. After MS2 exposure *AR*, *ITGB1*, *AKT*, *PGC1A* and *STAT3* were upregulated; *ITGA5*, *ITGAV*, *ITGB3*, *ITGB5*, *HSP90*, *MAPK1*, *MAPK3* and *PSA* were downregulated; whereas, no significant changes were observed in *HSP27* and *PI3K* (P value < 0.05).

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4.3 CHAPTER 3:**DEVELOPMENT OF PIPETTE TIP GAP CLOSURE MIGRATION ASSAY (S-ARU METHOD) FOR STUDYING SEMI-ADHERENT CELL LINES**

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Abstract:

This work presents a pipette tip gap closure migration assay prototype tool (semi-adherent relative upsurge—s-ARU—method) to study cell migration or wound healing in semi-adherent cell lines, such as lymph node carcinoma of the prostate (LNCaP). Basically, it consists of a 6-well cover plate modification, where pipette tips with the filter are shortened and fixed vertically to the inner surface of the cover plate, with their heights adjusted to touch the bottom of the well center. This provides a barrier for the inoculated cells to grow on, creating a cell-free gap. Such a uniform gap formed can be used to study migration assay for both adherent as well as semiadherent cells. After performing time studies, effective measurement of gap area can be carried out conveniently through image analysis software. Here, the prototype was tested for LNCaP cells, treated with testosterone and flutamide as well as with bacteriophages T4 and M13. A scratch assay using PC3 adherent cells was also performed for comparison. It was observed that s-ARU method is suitable for studying LNCaP cells migration assay, as observed from our results with testosterone, flutamide, and bacteriophages (T4 and M13). Our method is a lowcost handmade prototype, which can be an alternative to the other migration assay protocol(s) for both adherent and semi-adherent cell cultures in oncological research along with other biological research applications.

Keywords: Migration assay, Semi-adherent cell, Wound healing, Gap closure assay

5. GENERAL CONCLUSION

In conclusion, our work demonstrated that bacteriophages have natural tendency to interact with mammalian cells through their surface peptides and can influence the cancer cell growth and migration possibly by recognizing RGD binding sites on integrins.

- ⇒ It can be interpreted from bacteriophage T4 and M13 interaction with PC3 cells that bacteriophages significantly modulate genes related to integrins along with HSP and AR genes, which are important in cancer cell progression as inferred from histochemistry and cell migration studies.
- ⇒ On the contrary in the LNCaP cells, bacteriophage T4, M13 and MS2 interaction significantly downregulates most of integrin genes along with modulating important cancer cell progression genes and interfere in cell migration.
- ⇒ We successfully developed and reported a homemade pipette tip gap closure migration assay (s-ARU method) for studying migration assays in semi-adherent cell lines effective as an alternative to scratch assay.

It was observed from our studies that bacteriophage M13 was more effective as compared with phage T4 and MS2 in modulating crucial cancer cell progression genes as well as cancer cell migration and proliferation. Overall, bacteriophage M13 seems to be a promising candidate against both androgen dependent and castration-resistant prostate cancer and can be explored further for effective applications.

6. REFERENCES FOR INTRODUCTION

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ANNEXURE I**BACTERIOPHAGES IN CANCER CELL BIOLOGY AND THERAPIES**



Bacteriophages in Cancer Biology and Therapies

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Abstract

Cancer is one of the most challenging disease because of its unique individual identity and non-uniform progression within the same host. This confronting situation demands individual treatment therapies namely “personalized medicine”. Besides the improved success of available cancer treatments in the majority of cancer cases, for many of them, they have proved ineffective as the cancer cells evolve with treatment and show adaptive behavior. Bacteriophage (phage) therapy and phage-based therapies have gained special attention as a promising approach for cancer therapy beyond their action against multi-drug resistant bacterial infections. Considering the remarkable development in the phage research, mainly in biomedical sciences, molecular biology and nanobiotechnology; until now few conceptual reports about cancer and bacteriophage interactions and/or detailed study for phage-based therapies in cancer are available. These kind of information are valuable for reducing prejudices and to fundament discussions at regulatory agencies and departments for its safe use in humans for cancer therapy. Here we have tried to summarize the important works carried out about phage-cancer research and provide updated information about its ongoing progress and possible future perspectives.

Keywords: Cancer; Bacteriophage; Nanobiotechnology; Regulatory agencies

Abbreviations

AAVP: Adeno-Associated Virus/Phage; CTA: Clinical Trial Application; EMA: European Medicines Agency; GRP78: Glucose Regulated Protein; HAP1 Phage: A T4 sub-strain with a high affinity to melanoma Cells; HOC protein: Highly immunogenic outer capsid protein; HRP: Horseradish Peroxidase; HSP27: Heat shock protein 27; HSP90: Heat shock protein 90; IF: Interferon; IL: Interleukin; IND: Investigational New Drug; KGD Motifs: Tripeptide Lysine-Glycine-Asparagine Motifs; LNCaP: Lymph Node Carcinoma of the Prostate; PC3: Prostate cancer cell Line; PMN: Polymorphonuclear Leukocytes; RGD Motifs: Arginine-Glycine-Asparagine tripeptide; THPS: Tumor Homing Peptides; TSIPS: Tumor-Specific Internalizing Peptides; USFDA: United States Food and Drug Administration

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Introduction

Presently, various types of virus are used for broad range applications in biomedical fields including many mammalian and bacterial viruses (bacteriophages) [1-3]. Among them, bacteriophages have been explored for their range of applications since its discovery in 1915. Besides being a “rosette stone” for molecular biology [4], they have shown various promising application including phage therapy [5-8], phage display (expression of proteins or peptides on the phage capsid) [9-13] nanoparticles for practical applications in physical and material sciences [10], and industrial applications [14]. The rise of antibiotic resistant bacteria has increased our interest to focus on bacteriophages application as a potential treatment method for bacterial disinfection through phage therapy and phage enzyme applications [15]. There is a need for intensive research on bacteriophages and their role in cancer detection, treatment, diagnosis, etc. as reported recently by researchers worldwide [16]. The phages have been identified as a genetically modifiable molecule in various areas including medicine [17].

Considering the interest exhibited by various researchers through direct or indirect approaches for bacteriophage applications in medical sciences such as cancer research in last few decades, it is very essential to explore it further to know in which way bacteriophages may prove useful. The possible specificity of bacteriophage for certain cell and tissue components makes them a crucial candidate against cancer. Irrespective to the wide use of bacteriophages for their potential to kill many species of pathogenic bacteria through phage therapies; Nowadays, approaches for using bacteriophages against cancer cells are emerging a lot and further understanding on how mammalian cells responds

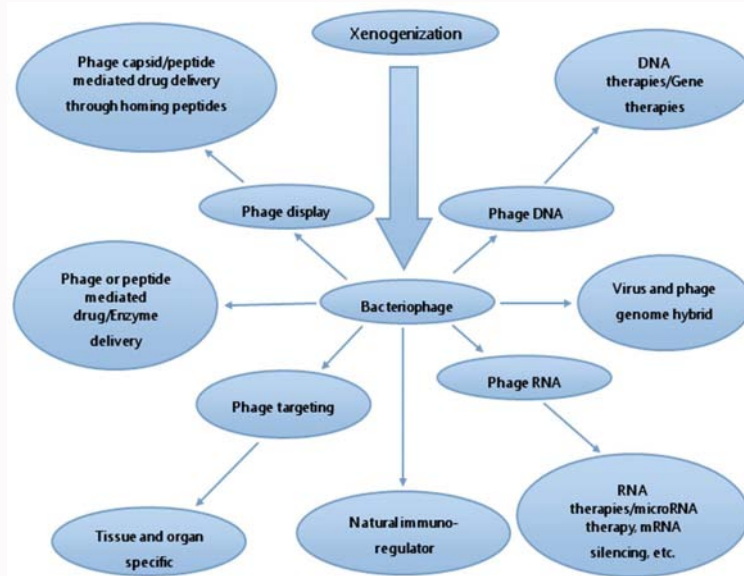


Figure 1: Ontology of bacteriophage applications for cancer biology and treatment.

in vivo to these phage agents with conjugates or through modification requires serious attention [18].

History of Bacteriophage in Cancer Research

Since, bacteriophages had proved to be one of the most important tools for various breakthroughs in molecular biology [19,20]. They are mostly studied for their derived applications worldwide along with their lytic life cycle which is well known and their lytic enzymes responsible for bacterial lyses events. The therapeutic potential of phages lies in their structure itself. Its minute and homogeneous size makes it the most capable nanoparticle for drug delivery as well as for various others purposes including phage display and targeting. Their capsid can be used directly to induce immune response naturally (due to their ubiquitous nature) or artificially (through genetic or protein engineering) in any organisms to express desired proteins on its surface to trigger immune response [21]. Due to the limitations of the current review article, we have restricted the broad applications of bacteriophages and just summarized the history of bacteriophage applications in cancer research and their possible outcome in Table 1.

Bacteriophage Interacts with Cancer Cell!

Although some studies have been conducted to know how the bacteriophages can interact with cancer cells through *in vivo* and *in vitro* studies, their impact on influencing host immune response as well as cancer cell physiology are less understood. In 1958, *in-vivo* and *in-vitro* demonstration of bacteriophage binding to the cancer cells was reported [22-24]. Similarly, the possible interactions between phage capsid proteins (KGD motifs) as well as β -1 and β -3 integrin receptors on target cancer cells was also confirmed along with the binding mechanism of bacteriophage T4 to Melanoma cells [25,26]. The most puzzling aspect of cancer cell is some of their behaviors, which are similar to a viral infected cell including apoptosis, cell cycle deregulation, metastasis, etc. [27]. Moreover, the 'oncogene', the genes which are attributed to most of the cancers have their origin from viruses. It is by now well established concept that cancer is a systemic biology disease and hence has a very deep root at the gene level [28]. Taking into account the inert properties of phages due to their non pathogenic nature for mammalian host, they can be

explored more extensively for further studies for their applications in cancer research.

Current gold standard treatment for cancer includes surgery, chemotherapy, radiation therapy and hormonal therapy. Other advanced treatment methods have shown promising response such as immunotherapy which includes tumor-targeting mAbs, dendritic cell-based interventions, peptide-based vaccines, immunostimulatory cytokines, immuno-modulatory monoclonal antibody, pattern recognition receptor agonists, immunogenic cell death inducers, etc. [29-33], are the emerging nanobiotechnologies recently developed for the detection and treatment of different cancers. Even though these methods are responsible for favorable response in cancer patients, patients now have the option for a personalized medicine approach [34], in which precise knowledge of the tumor molecular signature is accessible making personalized medicine mandatory [35]. In this scenario, bacteriophage, being a nanoparticle, can best fit to be a unique component for various molecular therapies and can be explored as a nanobiomedicine for cancer-treatment.

Why Bacteriophages for Cancer?

Bacteriophages are the most abundant entities in the biosphere that leads to its licensing for human use, mostly due to its non pathogenic nature and inert properties [36,37]. Being ubiquitous, we are frequently consuming large amount of phage populations through different sources [38]. Their detection in saliva, serum and stool samples confirms this finding and validates this observation [39-41]. Various results which have been published earlier showing wide applications of bacteriophages in cancer research and newly proposed applications seems to be very exciting (Figure 1). Some of the most preliminary and established bacteriophage applications are discussed below for getting an in depth idea about its current status and possible future applications.

Bacteriophage as a Natural and Involuntary Medicine

Bacteriophages seem to be an involuntary medicine as they are circulating in the mammalian body controlling invading pathogens and regulating our immune system naturally [42-44]. Furthermore,

Table 1: Time line for phage-cancer studies.

Year	Cancer Target/ Cell lines/Organism	Bacteriophage applications (Direct or indirect)	References
1940	Cancer tissue	Phage anticancer activity through accumulation for inhibition of tumor growth	(Bloch et al. [10])
1950-1958	Cancer cells	In-vivo and in-vitro phage binding to cancer cells	(Kantoch et al. [58]; Wenger et al. [110])
1970	Mouse	Purified T4 phage preparation administered intravenously to mice increased the level of interferon in animal sera.	(Kleinschmidt et al. [62])
1972	Tissue culture	Bacteriophages can influence human fibroblasts in tissue culture and induce immune response	(Merril et al. [72])
1979	In-vivo	Diagnostic or surgical procedures for cancer can result in metastases	(Krokowski et al. 1979)
1998-2002	Mouse	- Mapping of human vasculature by phage display - Cancer treatment by targeted drug delivery in a mouse model	(Arap et al.[3]; Arap et al. [2])
1999	In-vivo	Organ specific targeting using organ homing peptides and antigen detection approaches	(Pasqualini et al. [79])
2001	In-vivo	Phage can express genes from other organisms and do not multiply in mammalian cells.	(Di Giovine et al. [27])
2002	In-vitro	Biopanning and rapid analysis of selective interactive ligands (brasil)	Patent (WO 2002020822 A2)
2003	Cancer cells	Interaction between phage capsid proteins (KGD motifs) with Beta-1 and Beta-3 integrin receptors on target cancer cells	(Gorski et al. [40])
2004	Melanoma cells	Binding of bacteriophage T4 to Melanoma cells and its possible mechanism	(Dabrowska et al. [21])
2004	Solid tumor tissue	- Regulation of solid tumor growth with purified phage lysate - Non-purified phage lysate were reported to promotes tumor growth and development	(Boratyski et al. 2004; Dabrowska et al. [22])
2004-2005	Solid tumor tissue	- Comparative inhibition of metastases and solid tumor with T4 phage and HAP1 - Mutant HAP1 phage being more effective than T4 phage.	(Dabrowska et al. [22], Dabrowska et al. [23])
2005	human mono-nuclear cells	Purified T4 phage inhibits mitogen-induced IL-2 production by human mononuclear cells	(Przerwa et al. [83])
2006	In-vivo	Phages administered intravenously are rapidly phagocyted by liver cells.	(Gorski et al. [42])
2006	NA	Commercial phage preparations approved for human consumption by USFDA and phage-therapy products have been recognized for investigational new drug (IND) or clinical trial application (CTA) submission	(http://www.bioprocessintl.com/manufacturing/nonantibody-therapeutics/bacteriophages-an-alternative-to-antibiotics-challenges-and-possible-solutions-for-bringing-them-to-market/)
2008	Mouse MC38 colon carcinoma	Dendritic Cells increase their anti-tumor activity in presence of T4 phage (purified) and induces immune response against the MC38 mouse colon carcinoma and delay their growth.	(Pajtasz-Piasecka et al. [77])
2008	NA	The coining of "xenogenization" term for viral agents as an anticancer therapy.	(Sinkovics et al. [91])
2009	Mouse B16 melanoma	Bacteriophage T4 inhibited the adhesion of mouse B16 melanoma to fibrinogen and their metastases.	(Szczauraska-Nowak et al. 2009)

2009	Tumor tissue	Tumor specific phages can inhibit tumor growth, inducing the infiltration of Polymorphonuclear leukocytes (PMN), IL-12 (p70) and interferon- γ .	(Eriksson et al. [33])
2009	In-vivo	Peptide-mediated delivery of compounds deep into the tumor parenchyma using tumor-homing peptide- iRGD (CRGDK/RGPD/EC)	(Sugahara et al. [94])
2014	In-vitro	Bacteriophage M13 used for intracellular delivery of a functional enzyme (peroxidase - Horseradish Peroxidase - HRP) in PC3 cells (prostate cancer cell line)	(DePorter et al. [26])
2014	NA	Extracellular matrix composition for the treatment of cancers at molecular level involving different poly-peptides, mRNAs and microRNAs.	(Garzon et al. [37]; Ibrahim et al. [52]) US9034312B2 (US patent)
2015	Breast cancer	Development of RNA nanobiotechnologies (anti-miRNA) by using microRNAs for cancer suppression (eg. miR-21 for triple negative breast cancer)	(Shu et al., 90)

they can block $\beta 3$ integrin activity on neoplastic cells thus preventing growth and metastasis formation [42]. Moreover, they also restrict angiogenesis in the developing cancerous tissues or organs and prevent metastasis by inhibiting the adhesion of platelets and T-cells to the fibrinogens [42,45,46]. As initially, it was noticed that phage phagocytes is mostly occurs in liver and spleen [47], hence it can be said that being antigenic in nature they naturally behave as an antigen presenting cells and constantly act as a source for antibody production. Moreover, some phages have been observed to induce the secretion of interleukins (eg. IL-12) and interferons (eg. IF- γ) leading to the destruction of tumor tissues through cytokines; which initiate the cascade for neutrophil activation releasing reactive oxidative species (ROS) and other cytotoxic agents, ultimately leading to tissue damage [48]. In our ongoing studies, based on our preliminary results related to natural bacteriophage and Prostate Cancer cell (PC3) interactions it was observed that phages can stimulate internal cellular signaling and were responsible for gene expression alterations. The increased expressions for mRNAs corresponding to integrins (namely integrin αV , $\alpha 5$, $\beta 3$ and $\beta 5$) were detected significantly though quantitative PCR along with increase in AKT and PI3K signaling within these cells. Interestingly, we also got down-regulation for Hsp27 and Hsp90 genes after phage treatment, which are on contrary highly expressed in cancer cells and are responsible for migration, proliferation and prevention of apoptosis through survivin up regulation (unpublished). It was also observed from our studies that bacteriophage T4 and M13 interfere in the migration of PC3 and Lymph Node Carcinoma of the Prostate (LNCaP) cancer cells! (unpublished). This broad phage binding characteristic as well as its role in specific gene expression mostly through their proteinaceous external coat represents a natural way of phage display.

Bacteriophage for Gene Delivery, Gene Therapy, Cancer Detection, Targeting and Imaging

Recently, it has been showed that, bacteriophages can be harnessed for their potential in gene delivery to eukaryotic cells through transection of toxic genes to cancer cells. Such gene delivery

along with chemotherapeutic agents have great prospective in the near future [49-51]. Also, phage display is useful in cancer treatment because it is easy to modify the phage by gene insertion to express desired anticancer proteins and/or peptides on its capsids showing anticancer activities or induce immune response [52].

Similarly, various homing peptides have been reported against cancer specific cells, tissues and organs. The M13/T7 phages were used for tumor cell targeting through tumor homing peptides identification (eg. RGD-4C Peptide) on the cancer cells and tissue specific endothelial cells [53-58]. Moreover, organ specific targeting has been reported to be achieved by using organ homing peptides and antigen detection approaches (eg. prostate-specific membrane antigen) by using M13 as well as other phages [52,59-61]. It was also demonstrated that anticancer drugs efficiency against breast cancer was increased when homing peptides were used with integrins for drug delivery without much toxic effect [62,63]. Identification of tumour homing peptides and organ homing peptides can be helpful for targeting cancer cells, antigen detection and drug delivery using nanoparticles [61,62,64-66]. This approach facilitates the specific targeting of cancer cells, which can be optimized for targeted treatment or drug delivery through bacteriophages.

Nowadays, various databases and online tools are available for predicted, experimentally validated and manually curated comprehensive database for tumor homing and internalizing peptides (Table 2). Many Tumor-Specific Internalizing Peptides (TSIPs) or Tumor Homing Peptides (THPs) have been reported by using *in vitro* and *in vivo* phage display technologies; which specifically target tumor along with its microenvironments (eg., tumor vasculature) through their systematic delivery. Such internalizing peptides are of 3 to 15 amino acids length and are smaller than tumor homing peptides. Screening of proteins or peptides for protein-protein interactions can be carried out by displaying them on the surface of a phage through phage coat proteins for identification of internalizing or homing peptides [66].

Furthermore, promiscuous tumor targeting phage proteins have

Table 2: Databases and websites for tumor homing/internalizing peptides.

Database/Websites	Description	References
Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/main.php)	Innate immune peptides which also includes anti-cancer peptides.	Wang et al. [106]; Wang et al. [104]; Wang et al. [105]
TumorHoPe (Kapoor P et al. [59])	Manually curated comprehensive database with experimentally characterized tumor homing peptides which can be used to deliver drugs selectively in tumors.	Kapoor et al. [59]
CancerPPD (http://crdd.osdd.net/raghava/cancerppd/)	CancerPPD provides detailed information related to experimentally verified anticancer peptides (ACPs) and proteins.	Gautam et al. [38]
TumorHPD (http://crdd.osdd.net/raghava/tumorhpd/)	TumorHPD is a web server for predicting and designing tumor homing peptides. Such peptides are 7 to 12 residues short peptides having ability to recognize and bind to tumor cells or tissues; which can be used to deliver target specific drugs and as imaging agents for therapeutics and diagnostics.	Sharma et al. [89]
CPPsite 2.0 (http://crdd.osdd.net/raghava/cppsite/)	This database maintains experimentally validated CPPs which are unique cell penetrating peptides (CPPs).	Agrawal et al. [1]

been used for specifically targeting different cancer cells; It is reported that intercellular accumulation of phage clones in cytoplasm, mitochondria as well as nuclear region can be utilized as a potential ligands for intracellular drug delivery and/or molecular imaging techniques as they have few hours of pronuclear accumulation time [67].

Gene therapy is a technique in which functional gene is delivered or introduced into the target cells to restore, over express or inhibit desired gene products [68], which is now used to treat different cancers (e.g., peroxidases whose over expression can lead to the death of cancer cells). Similarly, telomerase is extremely dynamic in most of the cancer and fetal cells, but is almost undetectable in normal tissues hence can be applicable as a good tool for targeting cancer cells [69,70]. Furthermore, glucose regulated protein (Grp78) categorized within the heat shock protein (Hsp70) family can be used as a biomarker in stressed cells such as in tumors as a target for drug delivery or gene therapy by phages [71-73]. The chimeric phage vector, named Adeno-Associated Virus/Phage (AAVP) is a promising candidate in targeted gene therapy [74]. It is a hybrid of eukaryotic adeno associated virus and the filamentous M13 bacteriophage both having single stranded DNA. It expresses 3-5 copies of the cyclic RGD-4C ligand as in RGD-GC peptides on the phage pIII minor coat protein targeting $\alpha\beta$ 3-integrin receptor mostly associated with tumor cells [75,76]. Considering this scenario and potential of bacteriophages, they exhibit enormous prospectives in cancer detection, targeting and imaging.

Bacteriophage as an Immunomodulatory

The first study on interactions between bacteriophage and immune response were conducted by Felix d'Herelle demonstrating that phages have the potential to influence immune response [77]. Some phages like phi X174 are considered to be a standard antigen and can trigger humoral responses when administered intravenously; Since then, phage phi X174 have been extensively studied to evaluate humoral immunity in immunosuppressed patients [78,79]. Moreover, due to the omnipresent nature of bacteriophages in the biosphere, phage-neutralizing antibodies are reported to be present in the serum even before phage administration and with systemic administration can increase the phage neutralizing antibody titers [80-83]. Interestingly, considering the antigenic nature of phages, they can stimulate cellular response as whole phage particles and can induce stronger sensitization than their conjugate counterparts as reported in the case of phage sensitized lymphocytes in animals [84]. T-cells do not inactivate phages *in vivo*, which seems to be a very

good indication for further exploring phages for their potential as an immuno modulator [85].

Similarly, rodents show humoral response against phage coat proteins when introduced through oral route [86]. It was also reported that regardless of the route of phage administration, T4 phage particles are cleared considerably from the mice by spleen [86,87]. However, *in vivo* experimentation studies in mouse have led us to the observation that, T4 phage can be cleared from mouse circulatory system due to removal of Hoc protein (Highly immunogenic outer capsid protein) or defect in their genes [88,89]. As the results seems quite self-contradictory due to lack of more detailed investigation, there is a need for reevaluation and further in-depth studies for understanding this mechanism. Nevertheless, the therapeutic potential of the phages seems promising and demands urgent attention.

The most interesting thing to be explored is how phages modulate immune system of the diseased host (*in vivo*) and also cancer cell line responses (*in vitro*). The first report of phage demonstrating the immune response in animals dated back to 1956, have shown their potential as a new way of treatment methods in the near future [90]. The antibodies produced against phage T4 head proteins (i.e. Hoc and gp23) have been demonstrated to interfere in T4 phage activity [91]. Therefore, it is not a surprise to know that anti-phage antibodies are always present in the healthy individuals [90,92-94]. The studies on bacteriophages and their interaction in mammalian immune system are not yet explored thoroughly. Very few researchers have shown that phages can induce immune response due to their antigenic nature and many more accomplishments are yet to be made [95].

Patents on Bacteriophage Mediated Cancer Treatment

Recent increase in therapeutic approach for cancer treatment have facilitated the use of bacteriophages for cancer detection and treatment as various patents have been granted in the last few decades to phage based cancer technologies. The main aspect of developing the phage based technologies is not just restricted to its nanostructure, immunogenicity or ubiquitous nature but its flexibility, which can be utilized for different applications and therapies. Filamentous phages have been used as a modulator for angiogenesis (Patent: 20150110745) in which phages carrying bacterial lipopolysaccharide endotoxin on its capsid surface can be used for treating cancer by modulating angiogenesis process, which can be upregulated or down regulated depending upon the requirement for treating the cancer. Similarly, bacteriophages and their prophage proteins can be used *in vitro* to express poly-peptides having a proliferation inhibitory

activity in the mammalian cell lines as well as it can be used along with pharmaceutical compositions and gene therapeutic approach (Patents: US2009117084, US20050572260 and EP 1853291B1). A patent (US8507445) explaining the methodology as well as targeting peptide composition for diagnosis and therapy of human cancer was also introduced in last decade. Furthermore, patent (WO2002020822 A2) for biopanning and rapid analysis of selective interactive ligands (brasil) was also developed which helps for rapid and efficient separation of specific target binding phages.

Recent patent (Patent: US9034312B2) on extracellular matrix composition for the treatment of cancers looks very promising, as there are various reports of different poly-peptides, mRNAs and microRNAs interfering in cancer metabolic process at cellular and genetic level [52]. Even though few cytotoxic suicide genes are known, there is a requirement for an effective and efficient gene for target oriented killing of cancer cells in a patient (Patent EP 1618886 A1). If we are able to deliver a suicide gene within the specific cancer tissues or organs, nanoparticles like bacteriophage can assist us to specifically detect and target the cancer cells. Another example of combinatory therapy is using miRNAs, which have been reported to modify tumor phenotypes by regulating the genetic mechanism and also regulate cell growth and apoptosis [95]. Considering this potential applicability and possible approaches for targeting cancerous cells, bacteriophages proves to be a good candidate for cancer research encompassing various areas like cancer imaging, detection, targeting and treatment by both *in vivo* and *in vitro* applications.

Regulatory Framework for Phage Therapy and Bacteriophage-based Therapy

The bacteriophage therapy is been used for the treatment of various pathogenic bacteria as well as multi drug resistant bacterial infections and they have also shown potential applications for agricultural, animal and human use [96]. However, bacteriophage-based technologies are emerging significantly against various infectious diseases with the advancement in the biotechnological applications and provide options through improved phage-based therapies and diagnostics tools [97].

The main issue is in accepting phage based therapies or products for human use, which are restricted due to regulatory frameworks in different countries. In Poland, the clinical applications of bacteriophages are accepted for treatment as per Polish Law Gazette Number 28 of 1997 and Declaration of Helsinki [98]. Presently, the commercial phage preparations are approved against bacterial pathogens in food for human consumption as per USFDA and are restricted to phage therapy [101,103]. As per standard industrial production criteria, phage-therapy products have been recognized for Investigational New Drug (IND) or Clinical Trial Application (CTA) submission [99-102]. However, bacteriophage based therapies are not yet fully permitted for human use by some important regulatory bodies like United States Food and Drug Administration (USFDA) and the European Medicines Agency (EMA) as very limited randomized controlled clinical trials for bacteriophages have been conducted till date. As an alternative, some US companies have tried to develop phage based products for the decontamination purposes in agricultural sectors [103]. If the possible recommendation of phage based therapies are well accepted, it would be easier to have great awareness for accepting phage based products not only limited for use as an alternative for antibiotics, but also, for other phage derived products for humans including those for cancer treatments.

Future Prospective

The bacteriophages are presently used as a vector for various applications and have been reported to be important constituents of mammalian body playing a vital role in influencing their host immune response. As we know that extracellular matrix composition plays an important role in suppressing cancerous tumor growth, bacteriophages can be used to intensify this effect through their direct or indirect applications [104-109]. The phages can be used to display ploy-peptides and modulate useful constituent of extracellular matrix for anti-cancerous activities by regulating different poly-peptides, mRNAs and microRNAs interfering in cancer mechanisms. Similarly, phage mediated naked eye counting by miRNA molecule in petri dish can be implemented for detection of cancer biomarkers [110-114], which can prove helpful in understanding the cancer cell cycle and pathways. Recently, Anti-bacterial antibody-M13-Dye-probe was used to detect bacterial infections in living host, similar applications can be implemented for detecting cancerous tissues or organs in humans [115]. If we are able to optimize the therapeutic potential of bacteriophages for their direct or indirect applications, we can possibly revolutionize a whole new field of cancer treatment therapy. Bacteriophages can be an efficient cancer research tool and provide a better option for cancer treatment. As observed from the previous research works and results, the applicability of phages in medical field is still not fully explored and hence, in future tremendous breathtaking achievements and technological innovations awaits.

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ANNEXURE II**INTROSPECTING BACTERIOPHAGE SPECIFICITY AND DECODING
PHAGE ENZYME NON-SPECIFICITY FOR ANTIMICROBIAL
APPLICATIONS**

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4 Introspecting Bacteriophage Specificity and Decoding Phage Enzyme Non-Specificity for Antimicrobial Applications

4.1 Introduction

The Bacteriophages are still debated for their inclusion as a living or non-living nature by various biologists. The bacterial host specificity of phages is a matter of further research in the scientific community. The phage enzymes which have bacterial lytic properties have been studied for many decades by various researchers and produced a number of interesting findings that question the specificity of both bacteriophage host and enzyme specificity (Paunikar *et al.*, 2011; Sanmukh *et al.*, 2012; Khairnar *et al.*, 2013; Khairnar *et al.*, 2014).

Bacteriophages are reported to produce five major enzymes which include: peptidoglycan hydrolases, endosialidases, endorhamnosidases, alginate lyases and hyaluronate lyases, all have broad application potential (Drulis-Kawa *et al.*, 2015). The proteins encoded by lysis cassette genes viz. holins, endolysins, spanins are responsible for progeny release during the phage lytic cycle. The holin expressed in the late stages of phage infection forms pores in the host cell, allowing lysins to gain access to the peptidoglycan in the cell wall for the release of progeny phages (Loessner, 2005; Fischetti, 2004; Vincent, 2005). Phage lytic enzymes have been demonstrated to destroy bacterial cell wall, which has resulted in a new term “enzybiotics” for these proteins (Nelson *et al.*, 2001; Schuch *et al.*, 2002). The phage and phage-derived proteins have been explored for their various applications in therapy, diagnostics and biotechnology (Drulis-Kawa *et al.*, 2015).

The majority of endolysins have narrow range of lytic activity against bacterial cells when applied exogenously (Loessner, *et al.*, 1997) and its enzyme activity is restricted to the host bacterial species or genus specific from which it is derived (Zimmer *et al.*, 2002; Loeffler, *et al.*, 2001; Loessner, *et al.*, 1997, 2002). The broad spectrum lysins such as amidase PlyV12 (broad anti-bacterial range; Navarre, *et al.*, 1999) of *Enterococcus faecalis* phage Φ 1 exhibit anti-bacterial activity against *Streptococcus pyrogenes*, group B and C *Streptococci* and *S. aureus* (Yoong, 2004). Similarly, Mur-LH (muramidase) of *Lactobacillus helveticus* phage Φ 303 shows lytic activity against 10 different bacterial species (Deutsch, *et al.*, 2004). The other example includes *Streptococcus agalactiae* B30 phage lysin (Pritchard *et al.*, 2004) and *Clostridium perfringens* phage Φ 3626 Ply3626 amidase (Zimmer *et al.*, 2002).

The phage enzymes are highly evolved molecules which can quickly destroy the bacterial cell wall (Vincent, 2005). As described by Sulakvelidze *et al.* (2001), the lytic activity of phage enzymes is observed in growth media, phosphate buffer and even human blood. As the lytic activity is expressed in growth media, the effect of phage enzymes in combination can be more devastating for the pathogenic bacterial strains and can be used as a potential alternative or complimentary method for phage therapy.

Despite of the tremendous potential and wide range of applications of phage therapy, there is a need for further introspection about their nature and detailed mechanisms to enhance their uses. In the National Environmental Engineering Research Institute (CSIR-NEERI), research has been carried out on *bacteriophage therapy*. Phage therapy is widely used for the treatment of aquacultural pathogens, veterinary treatment and some are even approved for human applications (Sanmukh *et al.*, 2012; Khairnar *et al.*, 2013; Sanmukh *et al.*, 2014a, b; Khairnar *et al.*, 2014). Although the use of whole phage has the capability of generating the required enzymes continuously upon optimum nutrient and environmental conditions, the phages may undergo lysogenic conversions with their hosts and create a new problem of enzymatic resistance or provide phage immunity. Our major objective is to target the crucial component of phages that is responsible to carry out phage lytic activity, to avoid the involvement of the whole phage for the antimicrobial activity. We employed different lytic enzymes of the phages in consortia for the treatment of pathogenic bacteria. A new method has been developed to avoid the direct involvement of phages for the phage therapy by separate isolation and quantification of lytic enzymes against their specific hosts. In this chapter, we are going to discuss our new approach, which will benefit not only as a novel method but also it has proven to be less laborious, time efficient and effective.

4.2 Case studies

We selected six Gram-negative bacterial strains namely *Escherichia coli* (ATCC 15597), *E.coli* (ATCC 13706), *E.coli* (MTCC 1585), *E.coli* (MTCC 1588), *E.coli* (MTCC 521) & *E.coli* (MTCC 1650) for the study of phage lytic activity for antimicrobial application. The enrichment of different *E.coli* hosts was carried out in EC media (Eaton *et al.*, 1998).

The enriched host *E. coli* ATCC 13706 was inoculated with the ATCC 13706-B1 phage at 37 °C for 24 hours. In addition, enriched hosts MTCC 1585 and MTCC 1650 were separately inoculated with the MTCC 1742 phage at 37 °C for 24 hours (Standard methods, 1998; Paunikar *et al.*, 2012). The 1 ml of host (10,000 cfu/ml) was inoculated with 0.1 ml (1000 pfu/ml) of phage in 10 ml centrifuge tube, EC media (Eaton *et al.*, 1998) was used for each inoculation to make up the final volume 10 ml. The prepared samples were incubated at 37 °C till complete pelletization of host debris was observed in the each tube representing bacterial lysis (which is mostly 36–48 hours). The debris is an indication that the phage enzyme has effectively lysed the host cells resulting

into the release of new phage progenies in the broth. The broth now includes host debris, phage particles and phage enzymes. The debris can be separated by normal centrifugation at 5000 g for 5 minutes whereas, the phage particles can be removed by coal bed method in which phages are adsorbed to the coal bed and eluted afterwards with buffer. These enriched phages separated by our indigenous coal bed method (Dafale *et al.*, 2008; Khairnar *et al.*, 2014) are representing approximately the same concentration. The remaining filtrate will be just phage enzyme mixture. The filtrate can be stored in vials at 4 °C for further studies.

4.2.1 Procedure for studying antimicrobial action of mixture of phage enzymes

To study the effect of antimicrobial action of mixture of phage enzymes on the hosts the experiment was designed to facilitate the effective activity of lytic enzymes. The concentrations of the above hosts were so adjusted during pour plating so that the clear zone formation due to surrounding cell lysis can be seen. The EC media was added with 0.1 % 2,3,5-Triphenyltetrazolium chloride (TPTZ) solution for colour differentiation and lysis visualization for plaque assay (Eaton *et al.*, 1998) as shown in Figure 4.1. The concentration of bacterial consortia was 1000 cfu/ml. The crude enzyme mixture were added separately on two different plates to study the effect of its lytic activities (Fig. 4.2). The phage enzyme mixture can be added either by paper disc method or by bore well method for proper percolation of crude enzymes (Paunekar *et al.*, 2011; Khairnar *et al.*, 2014). We also kept control (plaques observed by using plaque assay method) to have a comparative study of both the methods.

We first tested the susceptibility of all six hosts for phage susceptibility by plaque assay method (Standard methods 1998). *E.coli* (ATCC 15597) and *E.coli* (ATCC 13706) were susceptible to the ATCC 13706-B1 phage, whereas *E.coli* (MTCC 1585) and *E.coli* (MTCC 1650) were susceptible to the MTCC 1742 phage. Out of the six hosts under consideration, *E.coli* (MTCC 1588) and *E.coli* (MTCC 521) showed no susceptibility for either phage. To test the non-specificity of phage enzymes, the consortia of six hosts were inoculated with phages separately to observe plaque formation as represented in Figure 4.1.

In our current studies, based on natural bacteriophage and prostate cancer cell interactions, we found that the bacteriophages can interact with mammalian cell surface proteins and can induce internal cellular signalling and gene expression alterations. After exposing 80% confluent (4×10^3) epithelial prostatic tumoral cell line PC3 to M13 and T4 bacteriophages for 24 hours with concentration of about 10×10^7 pfu (plaque forming unit), we observed that integrins, which includes integrin alpha V, alpha 5, alpha 3 and Beta 5; mRNAs expression were amplified significantly as analysed through quantitative PCR experiments. Moreover, bacteriophages exposure also induced an increase in the phosphorylated forms of AKT and PI3K by this cell, verified by western blotting.

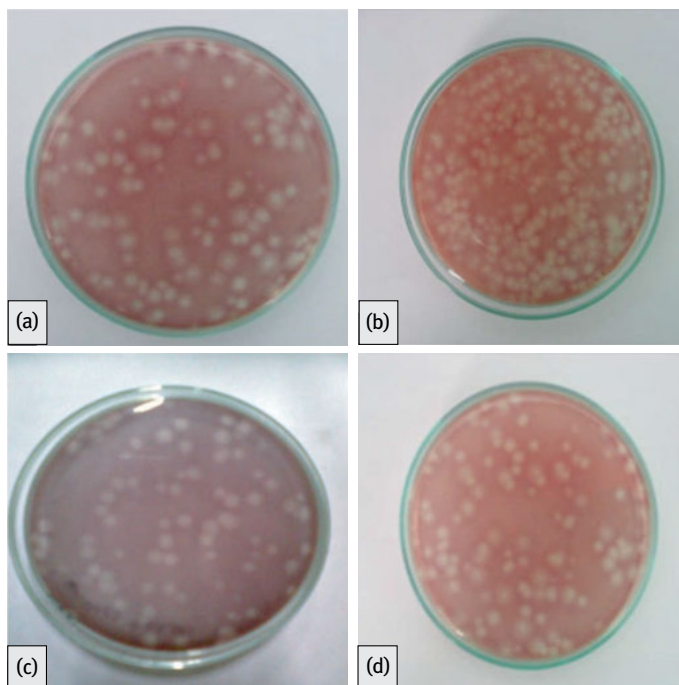


Fig. 4.1: The comparative study of plaque assay to show the non-specificity of phage enzymes as observed from plaque formation: **(a)** Mixed culture of *E. coli* (ATCC 15597) and *E. coli* (ATCC 13706) were susceptible to for ATCC 13706-B1 phage; **(b)** Consortia of *E. coli* (six strains), showing non-specificity of phage enzymes when inoculated against both the ATCC 13706-B1 phage and MTCC 1742 phage; **(c)** Mixed culture of *E. coli* (MTCC 1585) and *E. coli* (MTCC 1650) were susceptible to for MTCC 1742; **(d)** Consortia of *E. coli* (six strains), showing non-specificity of phage enzymes when inoculated against single ATCC 13706-B1 phage.

These effects on the specific gene expression reveals that phages have a broad range binding properties through their Proteinous external coat, which may be representing as a natural peptide display for inducing this effect. Moreover, previous works from Andrzej Gorski's group (Groski *et al.*, 2003; Dabrowska *et al.*, 2004(a); Dabrowska *et al.*, 2004(b); Dabrowska *et al.*, 2014) have verified similar properties in both in vitro and in vivo studies for melanoma cells. Our sole aim for conducting these study was to demonstrate that phages, in absence or presence of its host, have significant influence on their surrounding environment and, being a natural nanoparticles, are capable of demonstrating various novel properties, which we might had unable to identify.

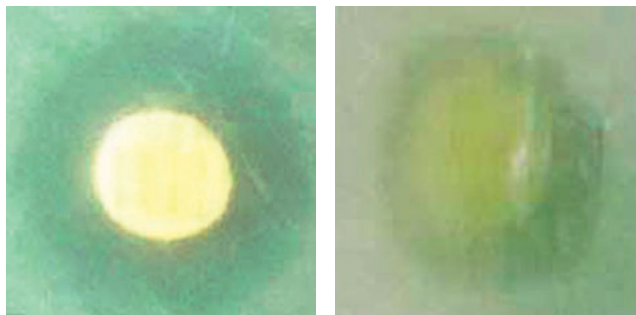


Fig. 4.2: The comparative study for understanding the lytic activity and efficacy of crude phage enzyme was demonstrated as observed from clear zone formation. Consortia of *E. coli* (ATCC 15597), *E. coli* (ATCC 13706), *E. coli* (MTCC 1585), *E. coli* (MTCC 1588), *E. coli* (MTCC 521) & *E. coli* (MTCC 1650) showing clear zone of lysis as observed from paper disc test due to lytic enzymes from ATCC 13706-B1 phage; MTCC 1742 phage.

Our preliminary results suggest that natural bacteriophages can interact with cell surface receptors integrins and mediate gene expression changes. Future *in vitro* as well as *in vivo* studies would be interesting to understand various properties associated with natural bacteriophages exposure (unpublished data).

Our work clearly aims for introspecting bacteriophage specificity and understanding phage enzyme non-specificity for antimicrobial applications for treating antibiotic resistant bacterial cells.

4.2.2 Results

The comparative assay for the study of lytic activities of phages and their enzymes demonstrated and verified the non-specificity of phage encoded enzymes as well as the anti-microbial activity exhibited by them. We found that by the use of paper disc method, the application of phage enzyme (crude enzymes) was quite easy, simple and effective as compared with phage plaque assay approach. The effectiveness can be observed from the clear zone formed around the paper discs represented in Figure 4.2. The result implies that the phage enzyme or combination of different lytic enzymes can be effective as they have activity against cell wall components. This clearly indicates that the crude or combined application of phage enzymes has more successful results as compared with the traditional phage therapy approach, considering its various advantages over phage therapy as mentioned in Table 4.1.

The quality and effectiveness of phage enzymes for their anti-microbial action can be optimized or increased by its selective purification and quantification. The

development of enzyme application method compared with phage therapy will boost the targeted antimicrobial action against any pathogenic or antibiotic resistant microorganisms. Establishment of this method will be helpful in assisting various other assays (such as comet assay, plaque assay and phage therapy) and for their modification as well as increasing their efficacy (Khairnar *et al.*, 2013; Khairnar *et al.*, 2014; Sanmukh *et al.*, 2012; Sanmukh *et al.*, 2015).

4.3 Discussion

In this case study, we have demonstrated comparatively the plaque assay method and crude phage enzyme approach for their potential lytic activity useful for antimicrobial applications against pathogens. The uses of phage enzymes directly have more advantages over the phage therapy (see Table 4.1) due to its minimum requirement and non-specific activity towards microbial cell wall. The susceptible host in the media was lysed due to the lytic activity of the phage enzymes; they also target the adjacent host cells which are not susceptible towards these phages. The adjacent host which have non-specificity towards the phages under considerations are targeted and get lysed due to the substrate specificity of the enzymes in action. The phages may be showing specific or broad specific activity towards their hosts, but the enzymes released followed by such lyses event are acting on the cell wall components of encountered hosts and are irrespective of their phage specificity.

Tab. 4.1: Comparison of traditional phage therapy and combined phage therapy approach for understanding its potential advantages.

Traditional Bacteriophage Therapy	Combined application of phage enzymes
Attachment with receptors for infection	Not applicable as enzyme is already released
Lysogenic formation within host	Not applicable
Phage resistance	Not applicable as phages are not involved
Environmental factors like temperature, pH, etc are determinative for proper infection and lyses event to occur.	Optimum temperature of enzymes, pH, etc is required for proper lyses event to occur.
A good nutrient condition is required for phage infection and lyses by phages.	Nutrient condition is not required as phages are not involved.
Complicated, laborious and time consuming	Simple, easy and efficient

The experiments conducted on consortia of different bacterial hosts and their phages showed the same results with delayed incubation time. This experiment thus demonstrates the collective activity of phage enzymes against microbial pathogens. As

endolysins are classified depending upon enzymatic specificity towards cell wall components (Loessner *et al.*, 1997; Young *et al.*, 2000, Loessner *et al.*, 2002; Loessner *et al.*, 2005, Fischetti, 2004), their combination will prove very helpful in combined application of phage enzymes against pathogens showing anti-microbial actions. It has been already reported that repeated exposure of such lytic enzyme(s) to bacteria did not lead to the resistant strains (Loeffler *et al.*, 2001). The lytic enzymes target the cell wall components and resistance has not been reported to these enzymes (Loessner *et al.*, 2005; Vincent, 2005). Hence, phage enzymes can prove to be very useful even for multi-drug resistant as well as antibiotic resistant pathogens.

Our experiment thus shows that the activities of phage enzymes are totally cell wall components specific and is independent of host type (Paunikar *et al.*, 2011) and reveal the combined application of phage enzymes is effective for controlling pathogenic organisms. It would be interesting to find out whether different phage enzymes can be used in combinations to inhibit pathogenic bacteria in natural environment and living organisms. The different phage enzymes varying in enzyme activity against various hosts can be applied in combination to lyse any pathogenic bacterial cell and thereby prevent outbreak of diseases.

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ANNEXURE III

**NATURAL BACTERIOPHAGES T4 AND M13 DOWN-REGULATES HSP90
GENE EXPRESSION IN HUMAN PROSTATE CANCER CELLS (PC-3)
REPRESENTING A POTENTIAL NANOPARTICLE AGAINST CANCER**

Natural bacteriophages T4 and M13 down-regulates *Hsp90* gene expression in human prostate cancer cells (PC-3) representing a potential nanoparticle against cancer.

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Short Communication

Prostate cancer (PCa) is the second most diagnosed cancer in male in the western world. In about 90% of the cases, it is potentially curable if detected early and treated. But, in the advanced metastatic stage, PCa treatment options are limited and exhibit a poor prognosis. Even though, nanotechnological approaches for cancer cell detection, targeting, and killing have increased; the challenge for developing effective and safe nano-material as nanoparticle still remains. Here we would like to shed light on “Bacteriophage” the naturally available nanoparticle, which have been effectively utilized for its anti-microbial applications for different pathogens and in other fields of biomedicine.

Our work includes the interaction studies between different bacteriophage strains with different PCa cell lines and to know how these particles modulate cancer cell behaviour *in vitro* (Figure 1). We would like to take this advantage to report our preliminary findings based upon the *in vitro* interaction studies from PCa cells (PC3) with bacteriophage T4 and M13. Our work was motivated from the previous works from Gorski's group, which have mainly highlighted mechanisms for phage-integrins binding specificity in cancer cells [1,2]. In our case, we are interested in the internal signalling and pathways associated with the direct interaction of T4 and M13 phages with prostate cancer cells (PC3). Our preliminary results have shown activation of some cell signalling pathways like PKB (AKT) and PI3K induced by natural bacteriophages interaction (Figure 2), very similar with fibronectin pathways [3]. We also have found that bacteriophage T4 and M13 significantly downregulates *Hsp90* gene (Figure 3), which is involved in cellular apoptosis, as well others cellular processes, indicating bacteriophages as promising candidate nanoparticle for cancer cell therapies.

As *Hsp90* is already reported as survivin suppressors [4], phages could be an effective tool in modulating survivin naturally or potentialized through phage display or other modifications. The survivin protein which has been reported as an anti-apoptotic protein represents smallest and last member of the Inhibitor of apoptosis (*IAP*) genes [5,6]. It is an important cell cycle protein, which promotes mitosis and DNA repair mechanism; whereas, inhibits apoptosis and autophagy along with its important function in mitochondrial repositioning and tumour invasion [7,8]. Use of a natural tools or vehicle as a carrier of proteins, peptides, oligonucleotides, and nucleic acids for anti-survivin therapy can prove far better than chemicals and synthetic drugs [4]. Bacteriophage and phage derived products can be an effective tool for developing such anti-survivin therapy, by suppressing

receptor *Hsp90* for example, which can be another option to cancer treatment therapies in the near future.

Moreover, bacteriophage interaction with cancer cells can induce others cell signalling pathways towards epigenetic changes. It has been shown that histone methyltransferase G9a is highly expressed in the metastatic prostate cancer cells and directly regulates the expression of several *Runx2* target genes, which function as critical regulators for the tumour growth, invasion and/or metastasis [9]. In addition, the recent study also indicates that histone lysine demethylases also play an essential role in the initiation and development of prostate cancers [10]. For instance, over expression of PHD finger protein 8 (PHF8) facilitates cell proliferation, migration, and invasion in prostate cancers [11]. Interestingly, as some of the histone modifiers are able to directly interact with the DNA methyltransferases (DNMTs) and play a role in the maintenance of DNA methylation at certain loci [12], DNMTs might also indirectly involved in the initiation and deterioration of prostate cancers. Extracellular vesicles have been reported to carry out epigenetic modifications in cancer microenvironment through DNA methylation/demethylation, histone modifications or non-coding RNA regulations (microRNAs and long non-coding RNAs) [13]. Similarly, bacteriophages/modified phages or their hybrids can be utilized for effectively down-regulating oncogenes expression.

Bacteriophage can also work as liposomal drug/peptide/protein delivery for targeting of cancer cells [14]. Similarly, bacteriophages complexed with synthetic nanoparticles had also got attention because of its hybrid nature, such as M13 with iron hydroxide [15]. More recently, f8 type of bacteriophages was introduced which are self-navigating phages, providing

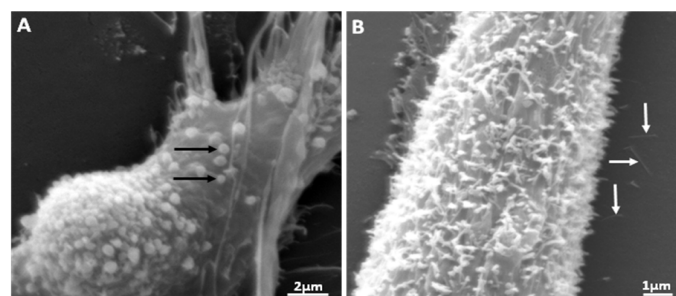


Figure 1. Scanning electronic microscopic image of prostate cancer cell (PC3) interacting with bacteriophage T4 indicated by black arrows (A) and bacteriophage M13 indicated by white arrows (B). Considering the small size of phage M13, we have pointed them outside the cell surface for having clear identification and resolution.

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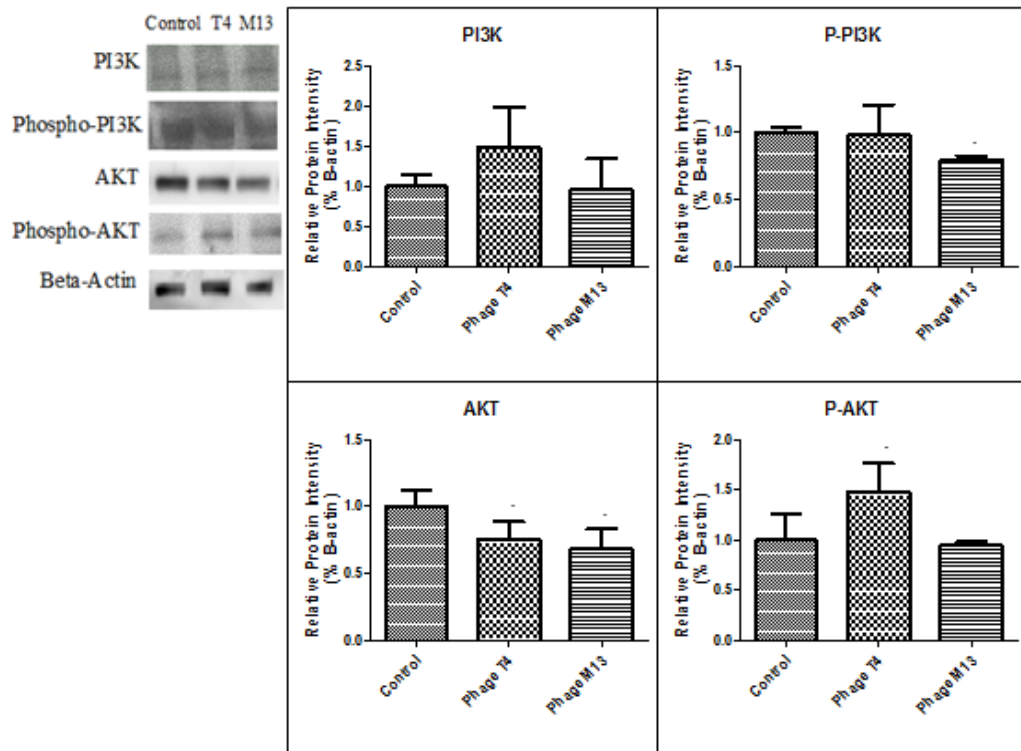


Figure 2. Representative western blot analysis for understanding the effect of phage interaction with PC3 on AKT (PKB) and PI3K protein, were analysed to determine the relative protein intensity (of % B-actin) after 24 hours exposure compared with their respective control. The PI3K, Phospho-PI3K, AKT and Phospho-AKT protein levels were normalized to β -actin levels. The intensity (Values are expressed by mean \pm SD, $p < 0.05$) were normalized to B-actin, which were set equal to 100% (equal 1). Protein levels differed significantly among the phage treated PC3 cells (* indicates $p < 0.05$, Turkey-Kramer multiple-comparisons test). The western blot band intensities were measured with ImageJ software. Note activation of P-AKT after phage T4 exposure.

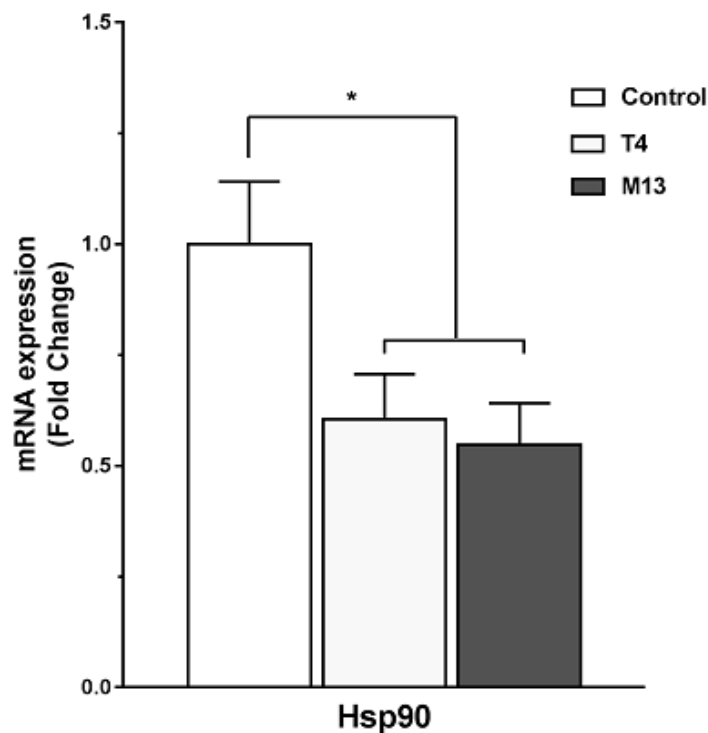


Figure 3. Relative expression of Hsp90 after interaction with bacteriophage T4 and M13 after 24 hours as compared with non-exposed PC-3 cells. Values are expressed by mean \pm SD, * $p < 0.05$.

multi-targeting ability which will certainly paved way for self-navigating nanomedicines for overtaking the homing and targeting barriers. In this sense, the combination of bacteriophage modified with RNAs, DNAs, peptides, proteins and synthetic molecules seems to be more promising approach against cancer cells as compared with other nanoparticles or chemically synthesized drugs alone [16].

Treatment option for the individual patient, based on the molecular profile of their disease, is the main challenge of modern personalized medicine, including PCa. Since bacteriophage is a naturally available nanoparticle and has no adverse immune response, our purpose for conducting this study was to demonstrate that phages are capable of influencing cancer cell signalling pathways and can be used to develop modified phage-based therapeutic products for cancer treatment [17].

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ANNEXURE IV

**BACTERIOPHAGE DERIVED PRODUCTS SHOULD BE ENCOURAGED AS
A DIRECT OR INDIRECT OPTIONS FOR DISEASE TARGETING-
PROMOTING AWARENESS FOR THEIR SAFER APPLICATIONS.**

Bacteriophage derived products should be encouraged as a direct or indirect options for disease targeting-promoting awareness for their safer applications.

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The continually escalating applicability of nanotechnological approaches for targeted treatment and therapies for various diseases, including cancer, demands, safety criteria supported by wide range of studies beyond ethical measures; showing its effective utilization without having hazardous effects on other cells than target disease. At present, many new nanoparticles have been synthesized which are demonstrating effective results against various cancers. Due to their effectiveness and manual control over its production their applications are well accepted, many are undergoing clinical trials and some have completed it successfully. Similarly, as per patents filed by various reputed research groups and companies, it seems that synthesizing new chemical compound followed by its patenting looks profitable [1]. However, we are sidelining an important nanoparticle which is naturally available and can be controlled in terms of production criteria for animal or human applications too! Bacteriophages are used in various fields including medicine for their various important applications (for example, phage display, phage therapy, phage-based drug/protein/peptide delivery) [2,3]. Even after been commercialized and licensed in 1940s by Eli Lilly and Company (US), for its use as a natural drug to treat bacterial infections; it has been undermined for other applications since the onset of the antibiotic era. Here, we would like to ask for our research community to encourage and demonstrate the awareness for its safe use as an option for internal medicines for targeting various diseases, such as cancer. In the last few decades, few studies reported the use of bacteriophages with modifications, hybrids or with chemical/metallic conjugates against few diseases and some types of cancers [2,4,5]. Moreover, the main concern about using bacteriophages in humans is related to its viral origin. Our only appeal to the publisher and to the researchers is to wholeheartedly report and support safer applications options for targeting different diseases by phage-based products or modified/hybrid phages. For promoting awareness about bacteriophages, its efficacy and efficiency through research articles, research notes, reviews, mini-reviews, short communications, commentaries, etc. is important for establishing its regulatory approval from crucial agencies like World Health Organization (WHO), United States Food and Drug Administration (USFDA) and European Medicines Agency (EMA) [6-8].

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ANNEXURE V**DEVELOPMENT OF PIPETTE TIP GAP CLOSURE MIGRATION ASSAY (S-
ARU METHOD) FOR STUDYING SEMI-ADHERENT CELL LINES**

Development of pipette tip gap closure migration assay (s-ARU method) for studying semi-adherent cell lines

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Abstract This work presents a pipette tip gap closure migration assay prototype tool (semi-adherent relative upsurge—s-ARU—method) to study cell migration or wound healing in semi-adherent cell lines, such as lymph node carcinoma of the prostate (LNCaP). Basically, it consists of a 6-well cover plate modification, where pipette tips with the filter are shortened and fixed vertically to the inner surface of the cover plate, with their heights adjusted to touch the bottom of the well center. This provides a barrier for the inoculated cells to grow on, creating a cell-free gap. Such a uniform gap formed can be used to study migration assay for both adherent as well as semi-adherent cells. After performing time studies, effective measurement of gap area can be carried out conveniently through image analysis software. Here, the prototype was tested for LNCaP cells, treated with testosterone and flutamide as well as with bacteriophages T4 and M13. A scratch assay using PC3 adherent cells was also performed for comparison. It was observed that s-ARU method is suitable for studying LNCaP cells migration assay, as observed from our results with testosterone, flutamide, and bacteriophages (T4 and M13). Our method is a low-

cost handmade prototype, which can be an alternative to the other migration assay protocol(s) for both adherent and semi-adherent cell cultures in oncological research along with other biological research applications.

Keywords LNCaP · Migration assay · Semi-adherent cell · Wound healing · Gap closure assay

Abbreviations

s-ARU	Semi-adherent relative upsurge
PC3	Prostate cancer cell line
LNCaP	Lymph node carcinoma of the prostate
ECM	Extracellular matrix
ATCC	American Type Culture Collection

Introduction

Cellular migration is an essential event for normal and abnormal physiological process, such as embryo development, angiogenesis, immune response, wound healing and cancer invasion and metastasis (Albini 1998; Fidler 2002; Wolf et al. 2003; Steeg 2006; Wyckoff et al. 2006; Hulkower and Herber 2011; Justus et al. 2014). Considering the importance of cell migration leading to metastasis in cancer, for example, understanding cancer cell behavior in vitro is an extremely useful tool from clinical research to drug

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discovery (Gupta and Massague 2006; Hulkower and Herber 2011; Kramer et al. 2013). Monolayer wound healing assays have been extensively used for studying changes in cytoskeleton rearrangement, cell-substrate adhesions and degradation, intracellular signaling pathways, nuclear reorganization, and gene expression (Savino et al. 2004; Belo et al. 2016). Also, integrins being the major family of migration promoting receptors (Ridley et al. 2003), their binding to ECM components regulates many signalling proteins involved in cell migration and invasion (Hood and Cheresh 2002). The migration assay is efficient for studying the roles of Ras superfamily of GTPases (Rho protein—Rho GTPases) in cell growth, migration, differentiation and in invasive behavior of carcinoma cells (Keely et al. 1997; Shaw et al. 1997; Allen et al. 1998; Itoh et al. 1999; Nobes and Hall 1999; Roberts et al. 1999; Banyard et al. 2000; DeVries et al. 1999; Burridge and Wennerberg, 2004). It is also reported that migration depends on activation of the ERK pathway and is independent of Ras activation (Anand-Apte et al. 1997; Heldin and Westermark 1999).

Due to its importance, many techniques have been developed for studying cellular migration and various companies have developed commercial kits or inserts for cell migration and invasion assays. The scratch assay or wound healing assay is the most commonly used non-commercial assays for various adherent cell lines, whereas Boyden chamber assayTM or MatrigelTM assay are used for studying the migration and invasion in adherent, semi-adherent and non-adherent cell lines (Kramer et al. 2013). Moreover, many commercially kits or inserts are available to ease such studies, including, Cell CombTM Scratch Assay (Merck Kenilworth, NJ USA.), ORISTM & ORISTM Pro Cell Migration & Invasion Assays (Platypus Technologies, Madison, WI, USA), RadiusTM Cell Migration Assays (Cell Biolabs, Inc., San Diego, CA, USA), CytoSelectTM Wound Healing Assay (Cell Biolabs, Inc., San Diego, CA, USA), Cell migration BiogelTM (Enzo Life Sciences, Inc., NY, USA), Millicell[®] μ -Migration Assay Kit (Merck KGaA, Darmstadt, Germany), QCMTM 24-well colorimetric cell migration assay (Millipore-Merck KGaA, Darmstadt, Germany), and IncuCyte[®] Scratch Wound Assay (Essen BioScience, Inc., Ann Arbor, MI, USA). All these available commercial products are useful and are being used for studying cell migration

and invasion assays. However, there are very few cost-effective options available for studying the migration assays (wound healing assays), especially for semi-adherent (like LNCaP) and non-adherent cell lines.

Considering the need for an economical alternative method for studying semi-adherent cell lines as well as their importance in oncological and other biological research, we report here the developed pipette tip gap closure migration assay (s-ARU method), for studying the migration assays for the semi-adherent as well as adherent cell lines. We applied this method to our studies with LNCaP prostate cancer cell line. The LNCaP cells are androgen-dependent epithelial cells and do not produce a uniform monolayer and are slightly attached to the substrate forming clusters (<http://www.lncap.com/>). They are slow growing and need more incubation time to grow after sub-culturing (<https://www.atcc.org/products/all/CRL-1740.aspx#characteristics>). We hope that our developed low-cost handmade method will prove useful and facilitate the research activities for other adherent and semi-adherent cell lines.

Materials and methods

Cancer cell lines, bacteriophage cultures, and other prerequisites

The prostate cancer-derived LNCaP and PC-3 cell lines (androgen-independent cell) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). The experiments were performed according to ATCC guidelines. These cells were individually seeded at 1×10^5 cells/well in 6-well culture plates and the experiments were carried out in triplicate. In case of the PC3 cell line, cells were washed with sterile D-PBS (GIBCO/Thermo Fisher). The recommended culture medium used is GibcoTM RPMI 1640 Medium (FBS free) (GIBCO/Thermo Fisher). All procedures were performed inside a 5% CO₂ incubator (Thermo Fisher, Waltham, MA, USA) under strict sterile conditions. The bacteriophage T₄ and M13 were purchased from New England Biolab's Inc (Ipswich, MA, USA). Testosterone and flutamide were purchased from MilliporeSigma (St. Louis, MO, USA).

Preparation of modified 6 well plates

For the preparation of modified well plates, six pipette tips with filter (of 1 ml volume each) were selected and shortened equally to the height of the wells with the help of a sterile cutter (or razor blade, 70% ethanol-treated). The tips were positioned in the center of each well and a drop of silicon glue was added over the filter of the tips. The cover plate was kept over the 6 well-plate with tips positioned at the center with glue at the top of the filter. The cover plate is left to dry. It has to be made sure that all 6 pipette tips have been fixed. For 1 ml pipette tips, the filter position provides a



Fig. 2 Representative image of modified-6-well culture plate prepared for s-ARU method migration assay. Shortened-1 ml pipette tips were fixed in the center of each well-cover using silicone adhesive, for creating a cell-free gap in the plate

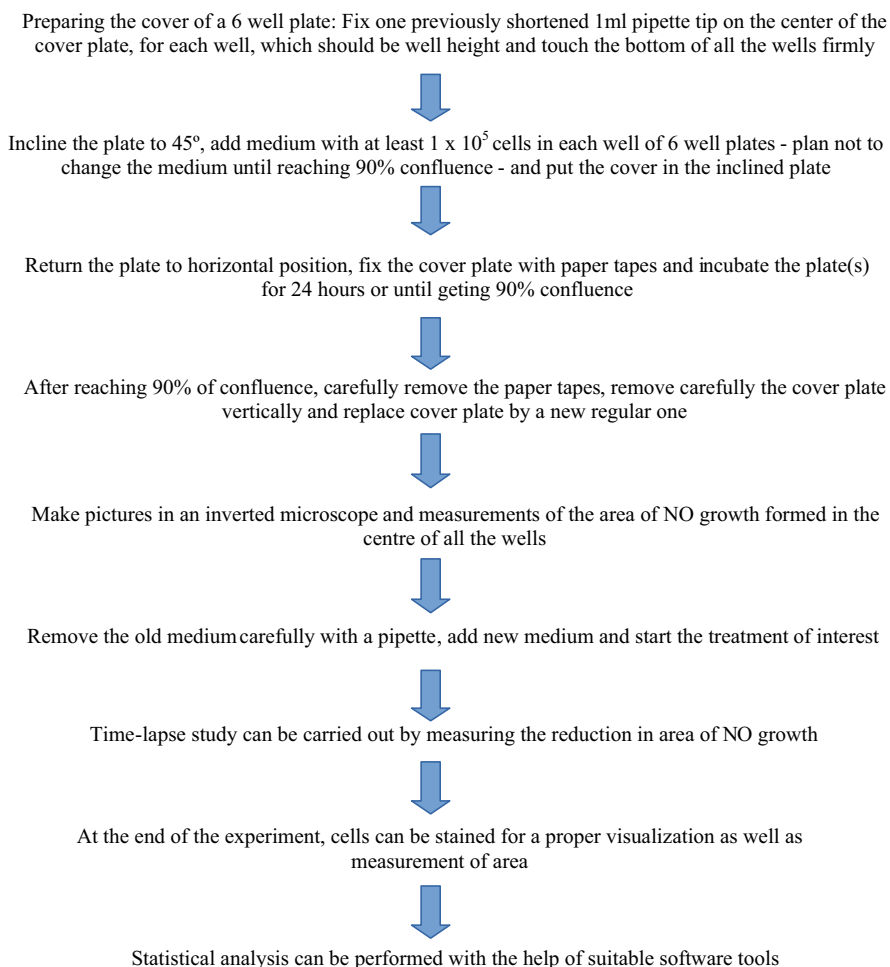


Fig. 1 Flowchart of pipette tip gap closure migration assay (s-ARU method)

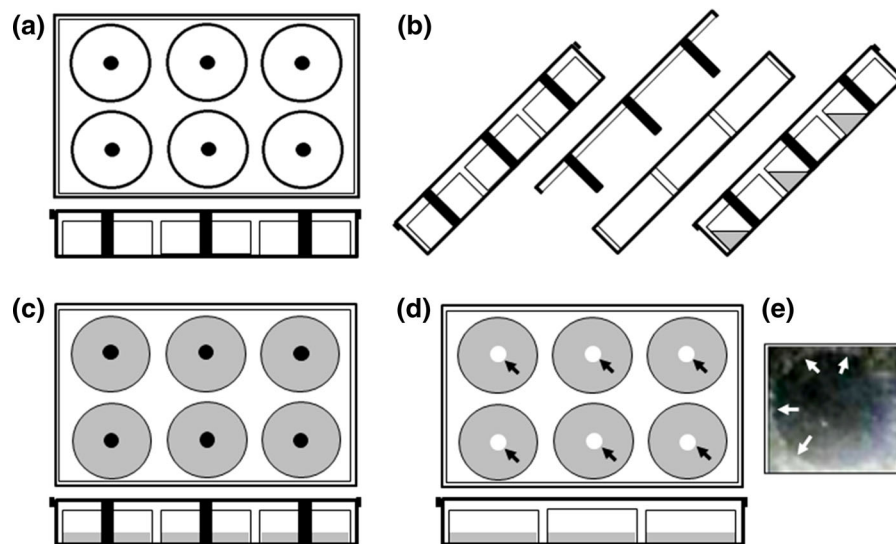


Fig. 3 Schematic representation of modified-6-well culture plate application in the s-ARU method for migration assay using pipette tips as a barrier. **a** Upper and lateral view of the modified-6-well culture plate. **b** Lateral view of the tilted modified-6-well culture plate for application of the medium-containing cells. **c** Upper and lateral view of modified-6-well culture plate in horizontal position, allowing cells to attach and to reach confluence. At this moment, cover plate needs to be fixed with

reference for cutting tips equally and the silicon glue will adjust and correct any difference in height. The directions for plate modifications are given in Figs. 1, 2 and 3. The plates can be UV sterilized to avoid any external contamination.

Inoculation of cells to grow and to reach confluence

With the plates tilted at 45° , RPMI medium mixed with 1×10^5 LNCaP cells were inoculated in each well of the prepared plates. The modified-cover plates with fixed tips were positioned with the plates still tilted at 45° , to avoid entering cells in the cell-free gap. The plates were returned to the horizontal position and the cover plates were carefully fixed with paper tapes. The plates were gently mixed and incubated for 24 h or until getting 90% confluence at 37°C .

Removing the modified-cover plate and measuring the cell-free area

After incubation, the plates were slowly rotated in one direction to detach loosely attached cells and then the

the help of sterile paper tape to restrict the free movement of the cover plate and preventing disturbance of the free-cell borderline. **d** Upper and lateral view of culture plate after cells have reached the confluence. A regular cover plate replaces the modified cover plate. The arrows point to the cell-free gap formed at the center of the wells. **e** Representative view of the cell-free gap formed by LNCaP cells after zone formation under normal condition. Arrows point to the borderline of the cell-free gap

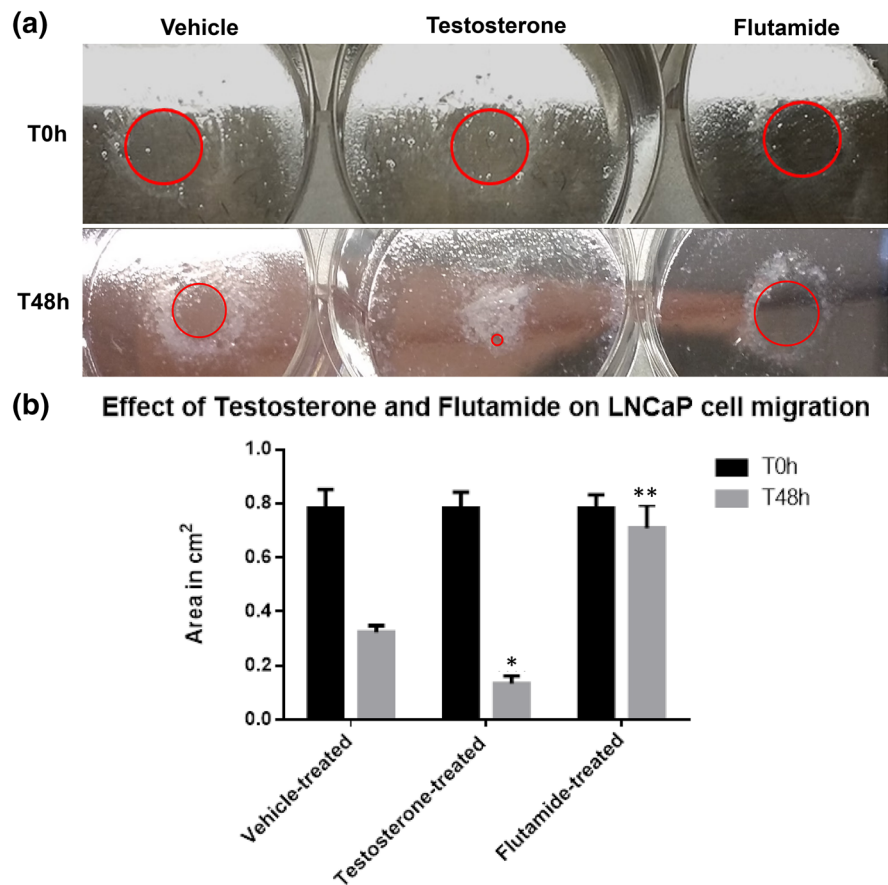
plates were tilted to about 45° to remove the paper tapes and the modified-cover plate. The medium along with suspended detached cells were removed with the help of a pipette (do not use the suction pump as it may disturb the weakly attached monolayer due to suction).

A new medium with the supplements for treatment were added to the wells and regular cover-plates were used for further incubations.

Testing the s-ARU method assay for studying LNCaP cells migration

The s-ARU method was used to study the cell migration assay for LNCaP cells. Confluent LNCaP cells were treated for 48 h with 10 nM of testosterone, 10 μM of flutamide, 1×10^7 pfu/ml of bacteriophage T₄ and M13, separately. For comparison, we also performed the scratch assay for LNCaP and PC3 cell lines. LNCaP and PC3 cells at 1×10^5 concentration were seeded in 6 well-plates. After reaching 90% confluence, scratch assay was performed for the LNCaP and PC3 cells. However, the LNCaP cells exhibited a remarkable irregular borderline after the scratch proceeding, producing significant differences

Fig. 4 a Representative image of LNCaP cells submitted to pipette tip assay (s-ARU method). After 24 h of plating the LNCaP cells and following removal of the pipette tip barrier, a spherical cell-free gap area could be easily identified (red circle), for all treatment, and it represents time zero hour (T0 h) or the beginning of the experiment. **b** Results of the measurements of the cell-free area (red circle) after 48 h (T48 h) of LNCaP cell growth and treatment with vehicle, testosterone and flutamide. The treatment with testosterone highly enhanced the growth and migration of LNCaP cell, while flutamide treatment was highly suppressive as compared with vehicle-treated cells (* means $p > 0.01$; ** means $p > 0.001$). The values are presented as the mean \pm standard deviation. (Color figure online)



in the initial gap area among the experimental groups and replicates. In this sense, it was decided not to proceed with this experiment for LNCaP cells. On the other hands, PC3 cells exhibited a uniform gap area among replicates and experimental groups and were then treated with 1×10^7 pfu/ml of bacteriophage T₄ and M13, separately. After 48 h the image of the wound was digitalized and the measurements of the area were performed.

Statistical analysis

The statistical analysis was performed by using two-way ANOVA with the help of GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, CA, USA) and ImageJTM software's (Rueden et al. 2017).

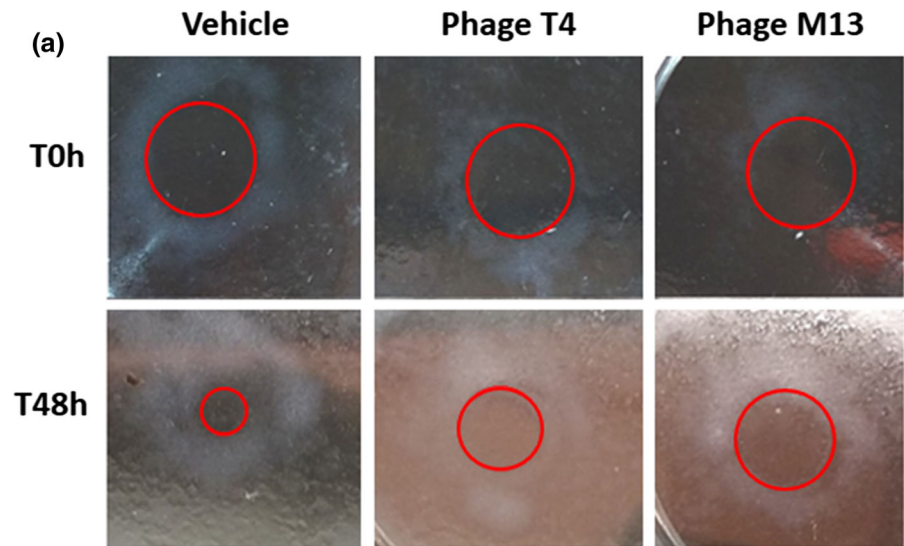
Result

The modified cover-plate with pipette tips attached was effective for creating a cell-free gap area in the center of the 6-well plate for LNCaP cells.

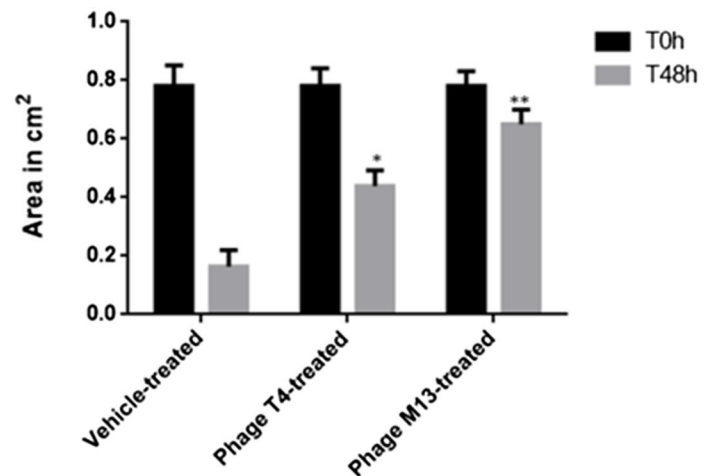
For testing and validating our s-ARU method, we evaluated the effects of testosterone and flutamide, an androgen receptor blocker, on LNCaP cells, which is an androgen-dependent prostate cancer cell line. As expected, testosterone treatment enhanced LNCaP cell growth and migration, whereas, flutamide treatment inhibited LNCaP cell growth and migration (Fig. 4).

We then applied our s-ARU method in another experiment. We were interested in evaluating the effects of bacteriophage T₄ and M13 on prostate cancer cells migration and proliferation, as were previously reported by Dabrowska et al. (2009) for melanoma cells and by Kurzępa-Skaradzińska et al. (2013) for leukemia cells. Here we performed a gap closure assay by exposing LNCaP cell line to two bacteriophages T₄ and M13 using s-ARU method. Our

Fig. 5 Representative image of LNCaP cells cultured using pipette tips as a barrier (s-ARU method). **a** After the incubation of LNCaP cells with pipette tip barrier for 24 h, the cell-free gap could be easily identified (red circle), for all treatment, and it represents time zero hour (T0 h) or the beginning of the experiment. LNCaP cells were exposed for 48 h (T48 h) to bacteriophages T4 and M13 at 1×10^7 pfu/ml concentration. **b** Results of the measurements of the cell-free area (red circle) after 48 h (T48 h) of LNCaP cell growth and treated with bacteriophages T4 and M13. Note that both phages reduced the rate of LNCaP cell migration, in which phage M13 (** means $p < 0.001$) was more effective as compared with the phage T4 (* means $p < 0.01$). The values are presented as the mean \pm standard deviation. (Color figure online)



(b) Effect of Phage T4 and Phage M13 on LNCaP cell migration



method showed that bacteriophage M13 has more anti-migratory effect on LNCaP cells as compared to bacteriophage T4 (Fig. 5).

Since PC3 prostate cancer cell line is an adherent cell, for our study with bacteriophage we performed the scratch/wound healing assay. The wound healing assay performed for the PC3 cells showed that bacteriophage M13 has an anti-migratory effect on PC3 cell lines as compared to bacteriophage T4 (Fig. 6).

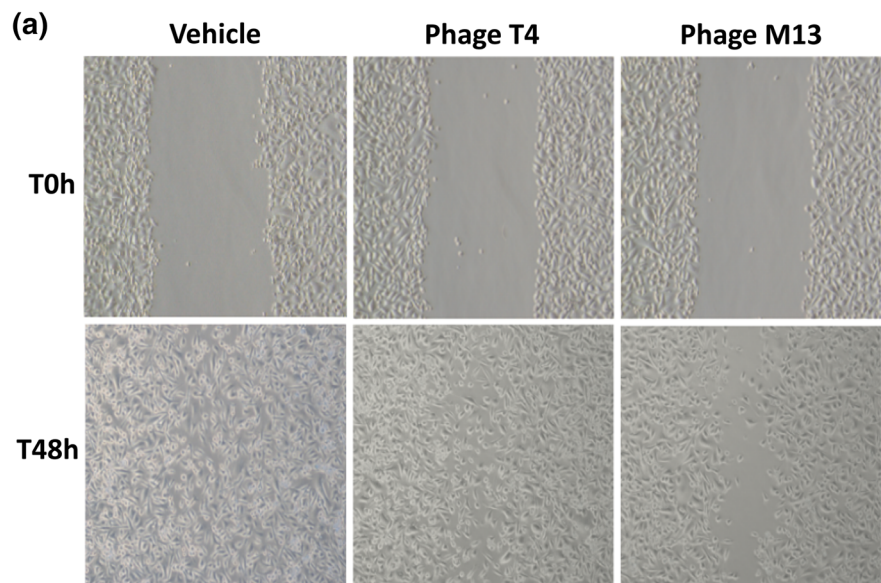
We performed a scratch assay for LNCaP cells also. As expected, LNCaP showed irregular and detached borderline and with remarkable differences among the groups and replicates, due to its semi-adherent nature

(Fig. 7). This aspect did not allow comparison of the initial gap area among the groups and replicates. In this sense, our pipette tip gap closure assay (s-ARU method) seems to be an effective alternative to the scratch assay method for this kind of gap closure assay for LNCaP cell line and others semi-adherent cells.

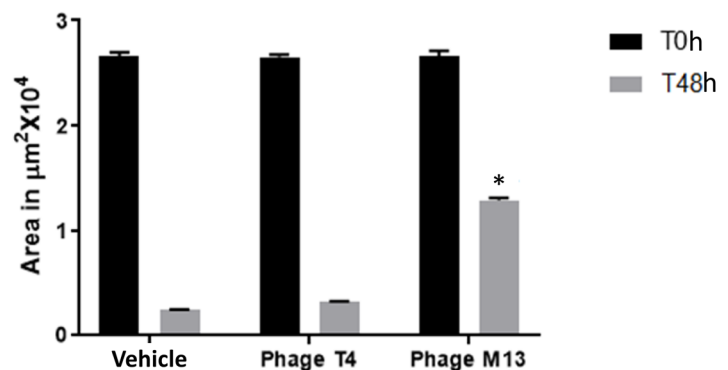
Discussion

Here, we presented a pipette tip assay (s-ARU method) to perform gap closure migration assay for semi-adherent cell lines. Our method can be applied to adherent cells lines as well, alternatively for

Fig. 6 a Representative images of the PC3 cells migration assay by scratch assay method. After reaching confluence, a scratch was performed (T0 h) and cells were exposed to 1×10^7 pfu/ml of bacteriophages T4 and M13 per well for 48 h (T48 h). **b** Results of the measurements of the cell-free area after 48 h. It was observed that phage M13 was effective (* means $p < 0.001$) in slowing down migration rate of PC3 cells and Phage T4 showed no significant effects. The values are presented as the mean \pm standard deviation



(b) Effect of Phage T4 and M13 on PC3 cell migration by Scratch assay



scratching assay. However, further modifications are necessary to minimize the limitations as listed in Tables 1 and 2. Our method seems to be an effective alternative to the available cell migration assays as most of them are ineffective in carrying out such assays for the semi-adherent cell lines and are just limited to adherent cell lines (Table 3) and can be implemented for studying other semi-adherent cell lines (Kramer et al. 2013). Biotech research companies for minimizing plate construction errors and maintaining uniformity in experimental proceedings can also utilize this prototype model for developing

low-cost modified cover-plate for commercial purposes.

Here, using the s-ARU method, we showed that bacteriophage T4 and M13 exhibited the anti-migratory effect on both PC3 and LNCaP cell lines, but bacteriophage M13 appreciably interfered in cell migration rates. The results of this study also corroborate the work of Gorski's group, in which they have studied the effect of bacteriophages on the migration activity of melanom with the help of purified T4 and HAP1 bacteriophages (Dabrowska et al. 2009; Kurzeņa-Skaradzińska et al. 2013). They have demon-

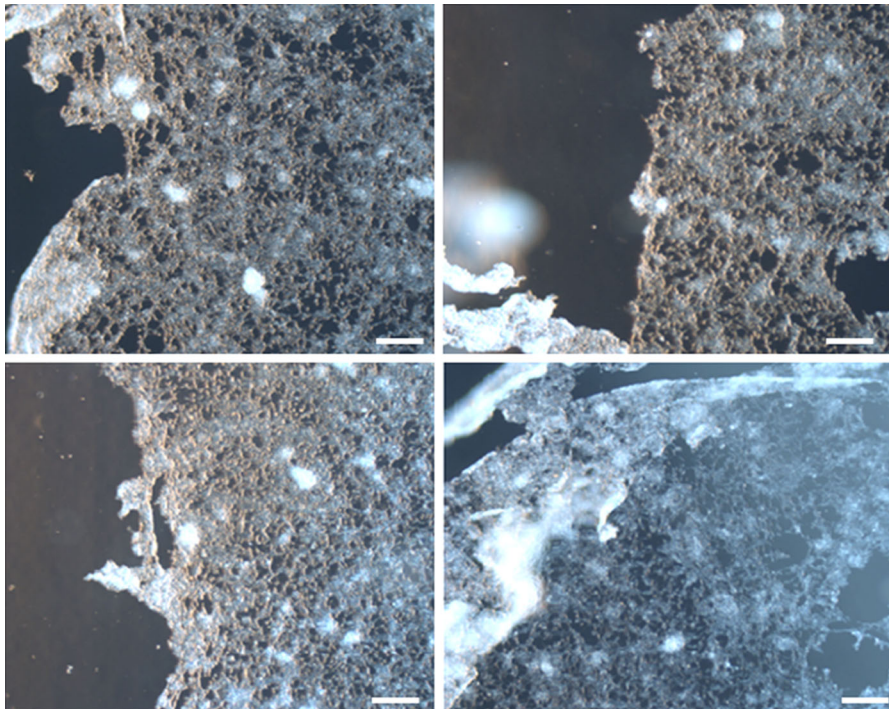


Fig. 7 The representative images of LNCaP cells after scratching for wound healing assay. In the scratch assay the monolayer borderline was disturbed and cells remained freely floating or loosely attached to the base of the plate, creating a

non-uniform and far different gaping area among replicates and experimental groups, and hindering measurements and statistical analysis. Note typical distorted monolayer borderline (scale bar = 100 μm)

Table 1 Advantages and dis-advantages of pipette tip assay (s-ARU) method

Advantages of pipette tip assay (s-ARU method)	Disadvantages of pipette tip assay (s-ARU method)
It can be used for adherent and semi-adherent cell lines	The use of pipette tips for building the modified cover plate unit is prone to human errors
Effective for carrying out toxicity test, drug assay and migration assay	It is time consuming (in the case of semi-adherent cells) when having multiple samples or compounds for analysis
It can be utilized for different microbiological and pathological applications (e.g. testing bioactive compounds against bacterial strains)	In case of semi-adherent cell lines, it is difficult to carry out time-lapse studies with the single plate and needs multiple units to be performed
As compared with the available methods, this method is consistent, reliable and effective	Due to improper operator handling, contamination can be a prime factor affecting final results
It is economical, user friendly and cost effective	Prototype is yet to be established to fulfill all the standards required for global acceptance and commercial production

strated that bacteriophages can interfere in the migration of cancer cells. It would be very interesting to explore further applications of natural and modified bacteriophages in therapeutic approaches for different types of diseases involving cell migration studies.

Our homemade pipette-tip gap closure migration assay (s-ARU method) was developed due to the difficulties associated with studying migration LNCaP cancer cell line because of its semi-adherent nature. Further improvements or progressive development involving this method are most welcome.

Table 2 Troubleshooting for the pipette tip assay (s-ARU method)

Problems	Solutions
Growth in the cell-free gap	- The pipette tip is not properly fixed in the center of the well or - Pipette tip is tilted or - Pipette tip is short
The cell-free area is not spherical/circular	- The cover plate is not fixed properly with paper tape or - The plate was tempered or - The medium and cells have entered under the pipette tip
Contamination	- The pipette tips are not sterile/non-autoclaved or - The pipette tips were not processed within the hood or - The glue/fixative/adhesive used might have contaminated the medium due to over spilling or shaking
The cover plate does not fix the plate properly	- The pipette tips are not homogeneously shortened or - The pipette tip is not properly fixed in the center of the well
Results variation	- The variation in results is mostly due to the density of the cells or - Floating cells occupying the NO growth zone

Table 3 Comparative analysis of different migration assays with respect to pipette tip assay (s-ARU method)

Cell migration methods/ techniques/ assays	Pipette tip assay (s-ARU method)	Trans-well migration assay (Boyden chamber)	Scratch Assay	Electric fence cell migration assay or ECIS	Spheroid migration assay	Capillary tube assays	Time-lapse cell tracking
Cell types supported	Adherent and semi-adherent cell cultures	Adherent, semi-adherent and non-adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures	Adherent cell cultures
Measurement	Area or diameter	Number of cells	Area	Area	Migration area	Migration tube distance	Cell migration path
Complexity	Extremely simple	Extremely simple	Extremely simple	Simple	Simple	Simple	Simple
Cost	Low	High	Low	High	High	High	High
References	–	Kramer et al. (2013) and Poujade et al. (2007)	Kramer et al. (2013), Liang et al. (2007) and Geback et al. (2009)	Kramer et al. (2013) and Cai et al. (2000)	Kramer et al. (2013) and Knupfer et al. (2001)	Kramer et al. (2013) and Young et al. (1999)	Kramer et al. (2013) and Zantl and Horn (2011)

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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ANNEXURE VI

PH.D. INTERNSHIP AT UNESP, BOTUCATU (ESTÁGIO DOCÊNCIA NA DISCIPLINA "MICROBIOLOGIA BÁSICA")

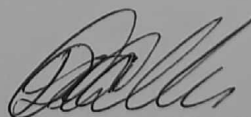
DECLARAÇÃO

Declaro para os devidos fins, que Swapnil Ganesh Sanmukh, atendendo aos termos da Resolução Unesp-4, de 22/01/97, realizou 45 horas de Estágio de Docência, sendo:

<i>Observação de atividades didáticas (Assistir aulas teóricas, práticas, seminários, etc...)</i>	<i>30 horas</i>
<i>Participação em atividades didáticas (Auxiliar e acompanhar atividades programadas para aula: exercícios, interpretação de texto, discussões de grupos, avaliações de seminários, etc...)</i>	<i>10 horas</i>
<i>Atividades extra classe (orientação de estudos e exercícios, preparação de aulas, etc...)</i>	<i>03 horas</i>
<i>Regência</i>	<i>02 horas</i>

O Estágio foi realizado na Disciplina "Microbiologia Básica", do Curso de Graduação em Ciências Biológicas (Bacharelado/Licenciatura), deste Instituto, sob supervisão do(a) Prof. Dr. Eduardo Bagagli, no 2º semestre de 2017, com início em 11/07/2017 e término em 07/12/2017.

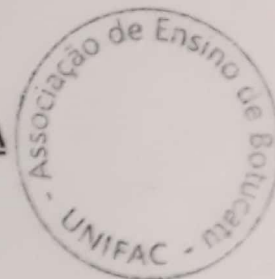
Botucatu, 22 de fevereiro de 2018.



Davi Barcellos de Oliveira Müller
Supervisor da Seção Técnica de Pós-Graduação

ANNEXURE VII

PH.D. INTERNSHIP AT UNIFAC, BOTUCATU (DISCIPLINA "EDUCAÇÃO FÍSICA E DOENÇAS CRÔNICAS")

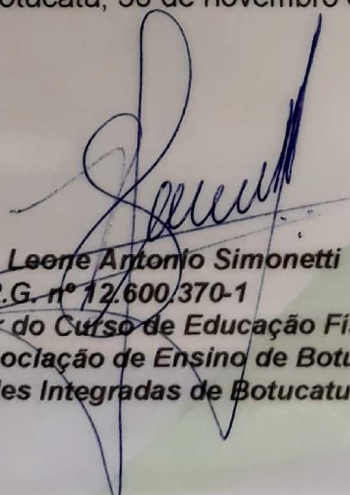
DECLARAÇÃO DE ESTÁGIO DE DOCÊNCIA


D E C L A R A M O S para os fins que se fizerem necessários que **SWAPNIL GANESH SANMUKH**, matriculado no Programa de Pós Graduação em Biologia Geral e Aplicada, área: Biomoléculas – Estrutura e Função, Curso de Doutorado da Universidade Estadual Paulista – Câmpus de Botucatu, desenvolveu atividades relativas ao **Estágio de Docência na Disciplina de EDUCAÇÃO FÍSICA E DOENÇAS CRÔNICAS**, sob a supervisão do Prof. Dr. Leone Antonio Simonetti, Docente e Coordenador do Curso de Educação Física – Licenciatura e Bacharelado, das Faculdades Integradas de Botucatu, mantidas pela UNIFAC – Associação de Ensino de Botucatu.

D E C L A R A M O S ainda, que o referido estágio desenvolveu-se no período de agosto a novembro de 2017, com carga horária total de 40 horas/aula.

Por ser verdade, firmamos e assinamos presente DECLARAÇÃO.

Botucatu, 30 de novembro de 2017.


Prof. Dr. Leone Antonio Simonetti
R.G. nº 12.600.370-1
Coordenador do Curso de Educação Física
UNIFAC – Associação de Ensino de Botucatu
Faculdades Integradas de Botucatu


Profª Gláucia Cristina Belver Vieira Alves
R.G. nº 23.962.601-1
Secretária Geral
UNIFAC – Associação de Ensino de Botucatu
Faculdades Integradas de Botucatu