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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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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*

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);

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

TABLE OF CONTENT

RESUMO

Sanmukh SG. **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** [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 analizada também por western blotting. Celulas 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 está interação com o bacteriófago M13 reduz a migração celular. Nossos resultados sugerem que os 3 bacteriófagos poderm 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*,

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).

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.

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