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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

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

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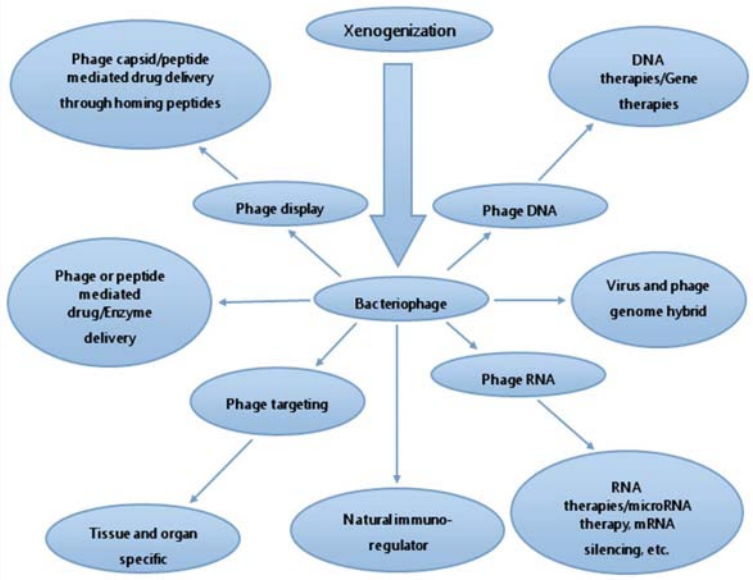

Figure 1: Ontology of bacteriophage applications for cancer biology and treatment.

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 *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, immuno-stimulatory 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	(Boratyjski 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.	(Szczaurska-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 β3 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-c) 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, α5, β3 and β5) 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 bio-marker 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 αvβ3-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 lipopollysaccharide endotoxin on its capside 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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