NOVEL PHAGES TARGETING INTRATUMOR ASSOCIATED *FUSOBACTERIUM NUCLEATUM*

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Introduction

Recent studies demonstrate that bacterial species are present within the tumor microenvironment (Geller et al., 2017). The presence of *F. nucleatum* in tumors has been proposed to increase cancer cell proliferation, promote chemoresistance and protect tumors against immune cell attack (Zhang et al., 2018). A higher abundance and prevalence of *F. nucleatum* has been associated with advanced tumor stage and poor prognosis in human colorectal carcinoma (CRC) patients (Mima et al., 2016, Yan et al., 2017). Moreover, *F. nucleatum* is maintained in distal metastases, demonstrating microbiome stability between paired primary and metastatic tumors (Bullman et al, 2017) Bacteriophage ('phage') are viruses that infect specific bacteria and play a critical role in regulating bacterial populations. Phages can be engineered to deliver therapeutic payloads (Schmidt 2019). Reduction of intra-tumor *F. nucleatum* and targeted delivery of anti-cancer payloads to tumors using phage may offer novel approaches for localized cancer treatment. To date there are only a few reports identifying phages which target *F. nucleatum* (Zheng et al., 2019). Using BiomX isolated phage against *F. nucleatum*, we have shown that these phages, when administered intravenously, can reach tumor-associated *F. nucleatum* (Kahan-Hanum et al., 2019). The aim of the current study was to isolate novel *F. nucleatum* infecting phages that may serve to decrease intratumor *F. nucleatum* burden and/or deliver a localized payload for anti-cancer treatment.

Methodology

- Natural phages were isolated from clinical samples, sequenced and tested against *F. nucleatum* strains.
- Phage sequences were analyzed following sequencing in Illumina using Nextera kits and genome assembly using SPAdes genome assembler
- Quantitative polymerase chain reaction (qPCR) was applied to detect *F. nucleatum* in CRC and adjacent tissues
- To study in vivo phage delivery, a syngeneic subcutaneous mouse model of murine colon carcinoma (CT26) was used
- *F. nucleatum* was injected IV and its presence in the tumor environment was determined by extraction, plating and colony counts (CFU)
- Phage delivery was carried out by a single IV administration and phage abundance in serum and in tumors was determined by counting plaque forming units (PFU) on lawns of *F. nucleatum*
- Phage editing was carried out by new molecular tools for *F. nucleatum* which were developed internally

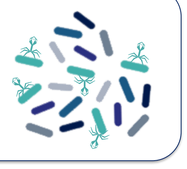
Background- Phage Properties

Safe IV administration

- FDA Workshop (July 2017) : "Bacteriophage are considered inert to human tissue"'
- Four decades of IV use to treat typhoid, reportedly with no cases of toxicity (Ochs et al 1971)
- Daily phage IV administration for 59 days did not result in any adverse effects (Schooley et al 2017)

Specificity

- Infect only specific bacterial strains



Immunogenicity

- In most clinical uses, phages promotes weak antibody response (Dedrick et al 2019, Żaczek et al 2016)
- In individuals with increased immune response, antibodies were not neutralizing the phage (Żaczek et al 2016)

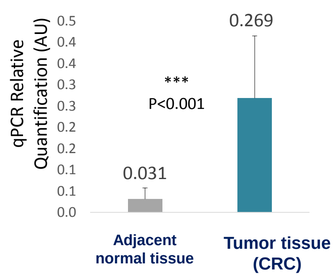
Results

1. High Prevalence and Abundance of *F. nucleatum* in Colorectal Cancer Tumors

Prevalence

Research group	No.	% positive	Detection method	Type
Li et al (2016)	101	87%	qPCR	Frozen tissue
BiomX	59	84%	qPCR	Frozen tissue

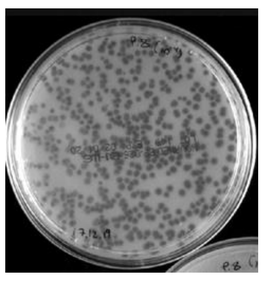
Abundance



Probe-based qPCR was performed to determine presence and abundance of *F. nucleatum* in colorectal cancer tissues. To quantitate relative abundance, levels of the F. nucleatum specific NusG gene (encoding a transcription antitermination protein unique *to F. nucleatum*) and of a human specific internal control gene (PGT) were measured simultaneously on DNA extracted with the Qiamp DNA Mini Kit (QIAGEN, Hilden, Germany). Samples were scored positive for *F.nucleatum* if $2^{-\Delta CT}$ was higher than 0.0002.

2. Novel Phages Identified Against *F. nucleatum* Subspecies

More than 10 novel natural phages for *F. nucleatum* were isolated and characterized for their host range on clinical *F. nucleatum* isolates. Both temperate and lytic phages recognizing different *F. nucleatum* subspecies (animalis, vincentii, nucleatum and polymorphum) were isolated. Two highly novel dsRNA phage were identified.



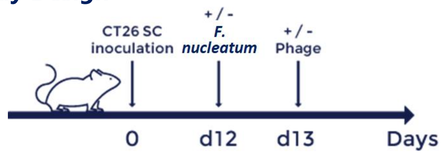
Plaques on lawn of *F. nucleatum*

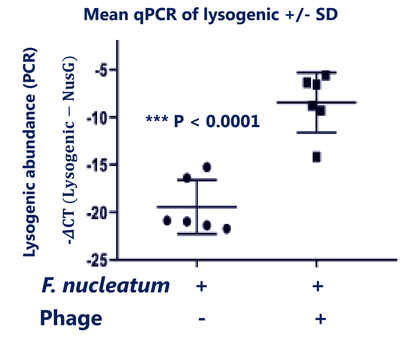
BiomX phage name	Genome type	Genome length (bp)	Phage type	*F. nucleatum* subspecies	Source of bacteria used for isolation
FN1-1	DNA	36,500	Temperate	Vincentii	Sinusitis
FN14-1p1	DNA	39,000	Temperate	Vincentii	Colonic tumor
FN14-1	DNA	110,000	Lytic	Vincentii	Colonic tumor
FN14-2	DNA	126,000	Lytic	Vincentii	Colonic tumor
FN14-3	DNA	80,000	Lytic	Vincentii	Colonic tumor
FN14-6	DNA	39,000	Temperate	Vincentii	Colonic tumor
FN2-58	RNA	8kb,5kb,3kb	Lytic	Vincentii	Inflamed gingiva
FN5-1	DNA	40,000	Temperate	Animalis	Colonic tumor
FN7-1	RNA	8kb,5kb,3kb	Lytic	Animalis	Normal colon biopsy
FN8-1	RNA	8kb,5kb,3kb	Lytic	Nucleatum	Colonic tumor
FNP-18	DNA	110,000	Lytic	Polymorphum	Human inflamed gingiva

3. IV Delivery of Phage to Intra-tumor *F. nucleatum*

To study if phages can reach and infect *F. nucleatum* embedded within the tumor microenvironment, we employed temperate phage whose incorporation into the bacterial DNA could be followed by qPCR with phage and host primers.

Study Design



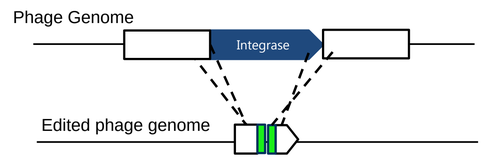


The abundance of the *F. nucleatum* temperate phage genome incorporated in the bacterial DNA was analyzed after normalizing to the total *F. nucleatum* content. *F. nucleatum* was determined by NusG quantitation using qPCR. Statistical analyses were performed using a two-tailed t-test. A statistically significant increase ($P<0.0001$) in temperate phage integration onto *F. nucleatum genome,* was observed in tumors derived from mice to which both *F. nucleatum* and phage had been administered compared to mice that received *F. nucleatum* only.

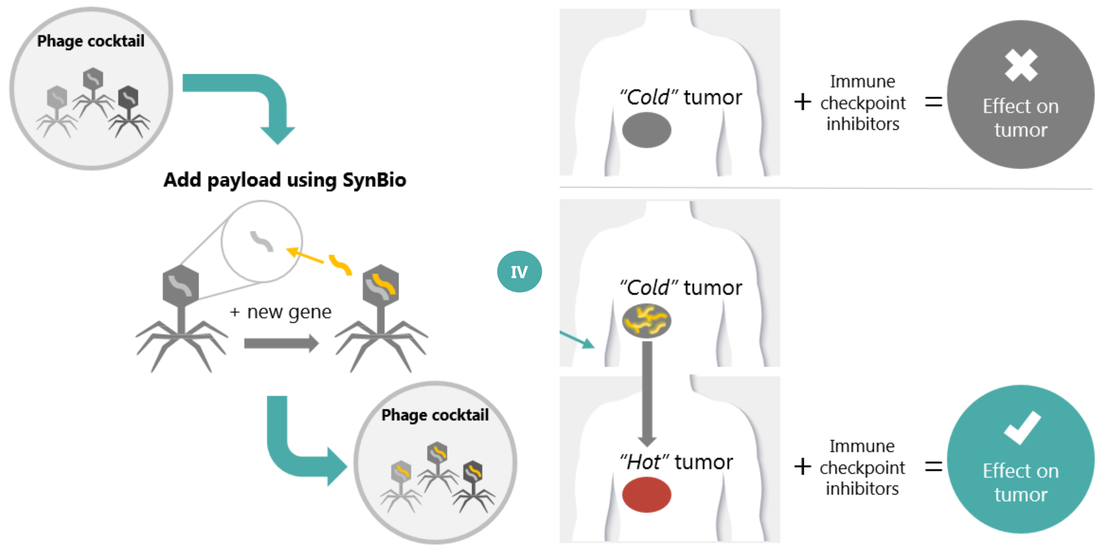
4. *F. nucleatum* Phage Engineered From Temperate to Strictly Lytic

A clinical *F. nucleatum* strain was successfully transformed and engineered, lytic phage was isolated (NGS verified).

Foreign DNA sequences were introduced into the phage, demonstrating the ability to add a payload.



5. Product objective: Engineered Phage Designed to Bring Immune-stimulating Payload to Bacteria in Tumors

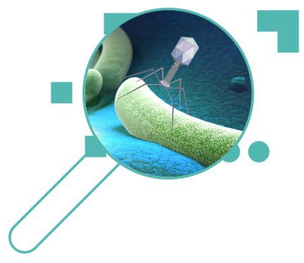


Discussion, Conclusions and Future Plans

- The newly discovered unique phages against *F. nucleatum* and successful conversion from temperate to lytic offer a first step in the development of a novel therapy targeting intratumor bacteria
- The phage will enable reduction of intra-tumoral *F. nucleatum* bacterial burden and delivery of a payload (i.e. anti-cancer or immune stimulating)
- The high specificity, low immunogenicity and safety of phage make it a promising agent to deliver heterologous anti-tumor payloads to the tumor microenvironment.
- The engineered phage will be coupled with checkpoint inhibitors to test improved treatment efficacy

References

Geller et al (2017)
Mima et (2016)
Bullman at al (2017)
Yang et al (2017)
Schmidt (2019)
Zheng et al (2019)
Kahan-Hanum et al (2019)
Li et al (2016)
Ochs et al (1971)
Schooley et al (2017)
Dedrick et al (2019)
Żaczek et al (2016)



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