NOVEL ANALYSIS OF *FUSOBACTERIUM NUCLEATUM* SUBSPECIES IN HUMAN COLORECTAL CANCER AND ENGINEERING OF THERAPEUTIC BACTERIOPHAGE

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Background

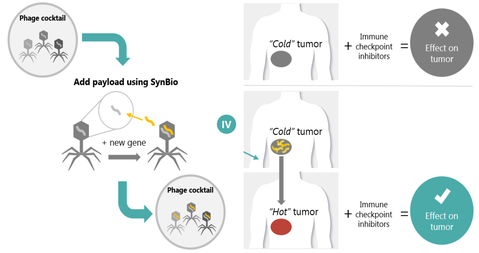
Recent studies demonstrate that bacterial species are present within the tumor microenvironment (Geller et al., 2017). *Fusobacterium nucleatum* (*F. nucleatum*) is significantly enriched in human colorectal carcinomas, (Ito et al., 2015) and its presence correlates with advanced tumor stage and poor prognosis (Mima et al., 2016, Yan et al., 2017).

Bacteriophage ('phage') are viruses that specifically infect bacteria in a subspecies specific manner. As they have no capability to infect mammalian cells they are considered to have a very high intrinsic safety profile.

We previously described the isolation of 12 novel natural phages targeting bacteria from all 4 subspecies of *F. nucleatum: animalis, nucleatum, vincentii, polymorphum* and showed the capability of phage to infect tumor-associated *F. nucleatum* in an animal model. Phage engineering would enable local delivery of therapeutic payloads to the bacteria-colonized tumor microenvironment as a novel cancer treatment, converting a "cold" tumor into a "hot" tumor when combined with checkpoint inhibitors.

To understand which phages could be useful in targeting intratumoral *F. nucleatum*, we performed analysis of the prevalent subspecies in CRC samples. In addition, initial steps in payload selection and phage engineering were successfully carried out.

Engineered phage designed to bring Immune-stimulating payload to bacteria in tumors



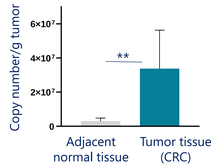
Results

1. High prevalence and abundance of *F. nucleatum* in CRC

Prevalence

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

Abundance (load)

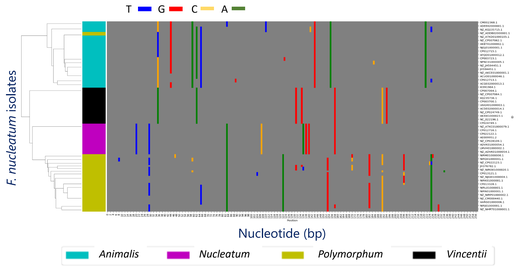


To quantify *F. nucleatum* presence and levels in colorectal cancer fresh frozen tissues, probe-based qPCR was performed measuring the level of *F. nucleatum* specific nusG gene normalized to a human specific internal control gene (PGT). Absolute quantification was calculated using a standard curve of *F. nucleatum* genomic DNA serial dilutions. The number of copies was normalized to1 g tissue, according to the weight of each sample (**p<0.05).

2. Targeted-NGS (T-NGS): a novel technology for *F. nucleatum* subspecies analysis in frozen tumors

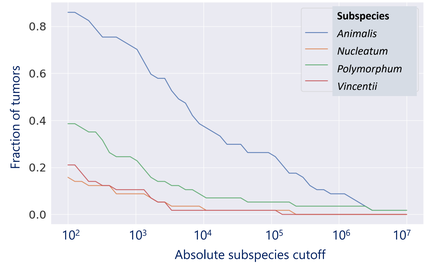
Because of the high level of phage specificity, in order to develop this novel approach for localized cancer therapy, it is necessary to understand the prevalence of *F. nucleatum* subspecies within the tumor. To discriminate between the 4 subspecies, an amplicon that yields different SNP patterns in each subspecies was identified and confirmed on 106 NCBI genome sequences. A novel algorithm was developed for classification of the sequences to the different subspecies following amplification and T-NGS.

Clustering genomic region of nusG gene



3. *F. nucleatum animalis* is the most prevalent and abundant subspecies in CRC

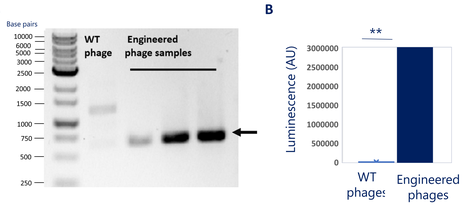
To identify *F. nucleatum* subspecies *in CRC* clinical samples, DNA was extracted from 57 frozen samples and subjected to T-NGS. The fraction of tumors that pass different cutoffs of absolute abundance for each species are shown in the below graph.



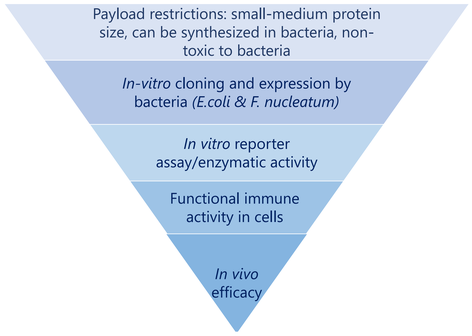
The analysis revealed that *animalis* was the most prevalent subspecies in all cutoffs. For example, at >10^2 cutoff, the prevalence of *animalis* was 86% (49/57), while that of other subspecies was lower; *polymorphum* 38.6% (22/57); *vincentii* 21.1%(12/57] and *nucleatum* 15.8% (9/57).

4. *F. nucleatum* phage engineering

To demonstrate that bacteriophages against *F. nucleatum* can be engineered to carry a payload, a sequence encoding an endogenous *F. nucleatum* protein fused to a labeled tag was inserted into the phage genome. Engineered phages were identified by PCR (A), and a luminescent signal was detected upon bacterial infection by the phages (B, **p<0.05).



5. Selection process for potential payloads



Aim: 2-3 payloads with different MoA in the final phage product

6. Candidate payloads

Three payloads were examined as candidates for phage therapy:

1. **GM-CSF-** induces anti-tumor immune responses by activating both innate and adaptive immunity.
2. **Cytosine deaminase (CD)-** converts the nontoxic prodrug 5-fluorocytosine (5-FC) to toxic 5-fluorouracil (5-FU).
3. **IL-15-** stimulates the proliferation and cytotoxic function of CD8+ T cells and NK cells.

The payloads were cloned into a *F. nucleatum* plasmid and were shown to be expressed by Mass-spectrometry. Next steps include engineering of the phage genome, and evaluation of *in vivo* expression and functionality in combination with checkpoint inhibitors.

Conclusions

The high prevalence and abundance of the *F. nucleatum animalis* subspecies in CRC samples suggests that it may serve as a target for phage-based treatment.

The high specificity and safety of phage make it a promising agent to deliver heterologous anti-tumor payloads to the tumor microenvironment.

Both reduction of intratumoral *F. nucleatum* bacterial burden and local expression of an anti-cancer or immune-stimulating payload to CRC by engineered phage may offer novel treatment approaches for patients with colorectal cancer.

Engineered phage may be coupled with checkpoint inhibitors to stimulate an immune response and increase efficacy.

References

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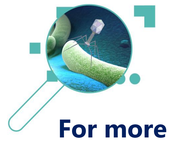

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