Microfluidic Inflammatory Tumor Model for High Throughput Drug Screening

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Background of research

The intricate interplay between the human genitourinary tract microbiome and the pathogenesis of genitourinary malignancies has emerged as a critical yet poorly understood facet of cancer biology. The tumor microenvironment, characterised by immune cells including tumor-associated macrophages (TAMs) and the presence of biofilms, is a pivotal focal point for understanding cancer progression and response to treatment. **However, the dualistic effects of bacterial-driven inflammation and the dynamic phenotypic shifts of tumor-associated immune cells and their possible treatment targets within this context remain areas of significant research interest.**

2.1 Portable microfluidics for point-of-care (POC) disease management: Due to the fast processing rate, small size, and multiplex assay capabilities applicable to even patient blood samples, microfluidics is important in advancing POC testing. *In vitro* drug screening platforms can benefit personalised medicine by integrating microenvironmental cues that mimic the tumor microenvironment. Capitalising on the portability and integrative capabilities of microfluidics, components can be introduced to mimic disease conditions *in vivo* for higher clinical relevance, allowing monitoring and prediction of outcomes in a routine and well-defined setting to resolve specific knowledge gaps in the research field. Automation of POC systems will also decentralise healthcare, standardise management, and reduce overall costs for disease management.

2.2 High incidence of systemic infections in patients with genitourinary malignancies: Most patients are highly susceptible to bacterial infections after chemotherapy, and inappropriate antibiotic treatment can worsen their prognosis and survival rates [1, 2]. Chronic inflammation caused by bacterial colonisation has been shown to play a key role in the origin and progression of cancer [3, 4]. The discovery of bacteria colonising in human pancreatic tumor cells was made several years ago [5]. However, detailed information about the impact of these interactions remains largely unknown. Bacterial infections are related to cancer development, especially in the digestive and urinary systems, which can cause chronic inflammation or produce carcinogenic metabolites, leading to cancer-promoting effects **(Appendix, Table 1)** [6, 7]. Clinical evidence shows that *Escherichia coli* and *Clostridium nucleatum* have carcinogenic potential in patients with colorectal cancer [8], and *Helicobacter pylori* infection is highly correlated with the risk of gastric and pancreatic cancer [9, 10].

2.3 Cancer Heterogeneity And Mortality: Cancer phenotypes can affect multiple signaling pathways, resulting in vast tumor heterogeneity [11]. The tumor microenvironment has been widely shown to induce tumor heterogeneity and enhance tumor progression [12]. The extent of tumor heterogeneity presents a problem during targeted therapeutics and is one of the main reasons cancer often leads to morbidity and fatality worldwide. This heterogeneity is also observed among cancer cells shed from the tumours [13, 14]. According to the World Health Organization (WHO), the projected mortality count for the next decade is 13 million [15]. Another major factor contributing to cancer fatality is the lack of early intervention, which often results in the developing patient metastases [16]. Most clinical cases were detected after cells from the primary tumor had migrated to other parts of the body [17].

It has been confirmed that different tumor types and tumor stages have different forms and locations of bacterial infection and colonisation [18]. Recent studies show that the disease status of cancer patients with systemic bacterial infections can be affected, which suggests that bacteria within circulation may also impact tumor progression [19-21]. Due to the intervention of antibiotics during cancer treatment, the common bacterial pathogens in cancer patients have gradually changed from Gram-positive to Gram-negative bacteria in recent decades, and they are more resistant to antibiotics [22]. As such, bacterial infections in cancer patients have become one of the severe complications during chemotherapy [23, 24]. Most cancer patients with sepsis are infected with Gram-negative bacteria [25]. From 2007 to 2014, the average recovery rate of bacteremia in this patient cohort was only 51.3% (24.7% -75.8%) [22]. As such, drug resistance caused by secondary infection has also become an urgent problem during cancer treatment.

2.4 Tumor models for drug screening: Despite the overwhelming evidence of the influence of bacterial infections on cancer patient outcomes, few studies have been conducted to evaluate new therapeutic strategies. One of the key reasons is that the mechanisms leading to these effects are currently under debate. Yet, the success of anti-cancer drug treatments hinges on the ability to predict and select the best combinatorial therapeutic agents while monitoring the outcomes of the treatment strategy.

The ability to translate research outputs of drug treatment studies involving *in vitro* models is still continuously questioned. Recent reports suggest using combination treatments with antimicrobial agents to improve outcomes. However, most models include 2D cultures of only cancer cell lines or bacteria cultures, which poorly represent bacteria-tumor samples. Although animal models are arguably more physiologically relevant to *in vivo* conditions, the results are often not translatable due to the different components of associated pathways between these animal models and human patients. As such, current tumor models cannot produce mutually consistent results, and both cancer-promoting and anti-cancer effects have been reported in the presence of bacterial-mediated inflammation [26]. Although many clinical studies have shown a correlation between the presence of bacterial infections and tumor progression [27, 28], a well-defined model is pivotal to revealing the detailed interactions between bacteria and tumor, which can provide adequate response measures for cancer patients with systemic bacterial infections, and reveal how the distribution of bacteria in tumor tissues can affect cancer cell phenotypes and tumor progression.

Previous related work by the team: The PI team previously described a microwell-based assay for patient-derived cancer clusters derived from liquid (blood) biopsy of breast cancer patients, with demonstrated relevance in predicting patient prognosis [29]. The assay combined parameters using microfabrication and hypoxia to mimic the tumor microenvironment *in vitro* [30]. The microwell array applies to cluster formation of other cancer types such as cervical, head, and neck [31]. **These parameters are the foundation for establishing this proposal's clinically relevant inflammatory tumor model.** Liquid biopsy contains circulating tumor cells (CTCs), which are shed from tumors. The presence of cancer cells in blood negatively correlates with patient survival [32-34], disease progression [35], and treatment efficacy [36]. Reports also associate primary CTCs with heightened drug resistance [37] or tolerance [38] traits. Using the microwell-based assay, the CTC clusters were positively screened for six target genes (FGFR1, Myc, CCND1, HER2, TOP2A, and ZNF217) associated with breast cancer, and the heterogeneity of gene expression was observed across patient samples [29]. Phenotyping of CTCs reveals the presence of cancer stem cells (CSCs) [39], which also originate from tumors and may present heightened tolerance [38] or resistance [37, 40] to specific chemotherapeutic drugs. These subpopulations of cancer cells with favorable traits for survival are valuable drug targets for novel therapies.

Preliminary studies demonstrate that the microwell arrays can establish uniform co-cultures of bacteria-tumor clusters under defined conditions (**Figure 1**). The microwell-based assays enabled the formation of 3D structured biofilms, which are more representative of biofilms *in vivo* than traditional 2D models [41]. **Together with the analysis of CV intensity based on OD595 absorbance values and the structural analysis from scanning electron microscopy (SEM) images, we could confirm that the presence of biofilms is specific to the CT model**. Co-cultures were established with bladder cancer cell lines (UMUC3) and pathogenic *Escherichia coli* (UTI89). Urinary tract infection (UTI) by uropathogenic *E. coli* (UPEC) infection has been proved to be a contributing factor to the development of bladder cancer [42, 43].

Tumor clusters remained viable prior to the onset of infection. The cells were uniformly suspended in the growth media and seeded into each microchannel at the optimal concentration, forming uniform clusters in the center of each microwell after 24 h and could be processed for downstream analysis. Clusters can be characterised by live/dead staining, immunostaining, crystal violet staining, and colony-forming unit (CFU) quantification to investigate tumor response (**Figure 2**). Spatial distribution of the bacteria within the tumor appears to impact tumor response, as reflected by the higher cluster adherence observed with extratumoral bacteria (EB) but not intratumoral bacteria (IB).

Tapered morphology for microwells to establish uniform co-cultures is crucial for drug screening consistency and has been validated with new drug combination screening [45]. Cylindrical microwells led to multiple irregular small clusters of ~10-20 cells, while tapered microwells consistently formed single large clusters comprising ~50 cells (**Figure 3**). Through the model, we validated a new combinatorial strategy of PDT and chemotherapy by co-delivering a bacterial-targeted photosensitiser with aggregation-induced emission (AIE) property and an anti-cancer drug, doxorubicin [46]. The effect of combinatorial therapy was synergistic, resulting in improved efficacy, as evidenced by at least a 2.5-fold reduction in the half-maximal inhibitory concentration of doxorubicin.

In the last decade, the co-PI team have been working on multidrug-resistant bacteria in urinary tract infections, mainly focusing on disseminating drug-resistant genes through horizontal gene transfer and determining factors that provide survival advantages to bacteria under stress environments (figure x). Moreover, we have identified several key elements in virulent plasmids that contribute to the fitness and physiology of bacteria, particularly those small RNA (sRNA), which is known to play the master role in regulating numerous important pathways in response to environmental changes during infection such as colonization and invasion. Nevertheless, the effect on the bladder cancer cells remains unclear.

We envisage that our proposed study will promote a better understanding of the impact of bacteria on tumor progression and that the model can facilitate drug discovery of antibacterial agents for biomedical and pharmaceutical industries. **The efficient automation of these quantitative components will provide us with novel means to screen patient responses in a cost-effective and non-invasive manner.**

Table 1 Impact of inflammation on cancer development and progression.

Cell line and animal models				
Type	**Cell type**	**Bacteria**	**Cancer-promoting effects**	**Ref**
Breast	MDA-MB-231	*Staphylococcus aureus*	Promote invasiveness and adhesiveness of cells	[1]
Prostate	RWPE-1	*Propionibacterium acnes*	Induce inflammatory response; Lead to malignant phenotype transition	
Clinical studies				
Colon	NA	*F. nucleatum*	Promote angiogenesis; Recruits tumor-associated immune cells	[2]
Gastric	NA	*Helicobacter pylori*	*C*auses cell DNA damage	[3]

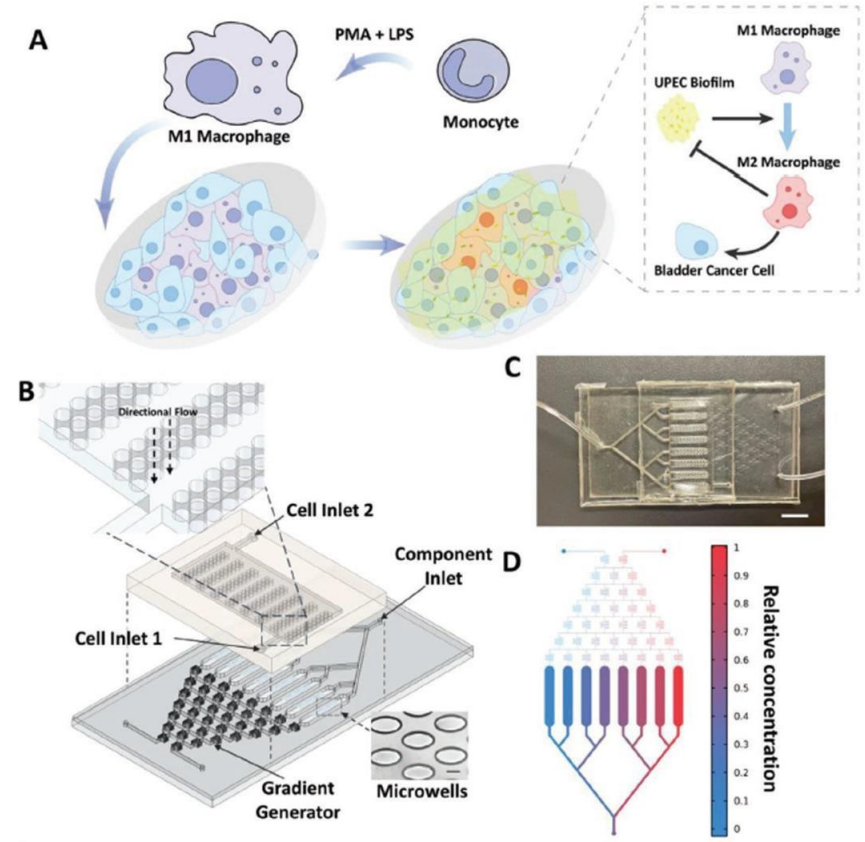

Figure 1: Development of a TAM-based microfluidic inflammatory tumour model. (A) The design of the device consisted of three main components. (B) Photograph of a PDMS device filled with a gradient of food dyes. (C) A gradient of concentration in the gradient generator simulated by COMSOL ® Multiphysics. (D) The schematic illustration of bacteria and cell culture with treated drug, and (E) the final established *in vitro* 3D IB and EB model.

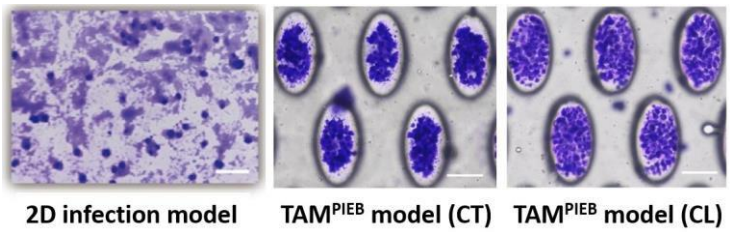

Figure 2: Clinical relevance of the model. Comparison of E. coli biofilm formed in a 2D infection model (left) and the TAM^{PIEB} device (right), the latter of which is more clinically relevant to in vivo infections. Spatial distribution of the bacteria within the tumour influences tumour response, as reflected by the higher cluster adherence observed with extratumoral bacteria (left) but not with intratumoral bacteria (right) at 1 h after infection at the low MOI of 1:1, as indicated by the crystal violet staining. Scale bar, 100 μm.

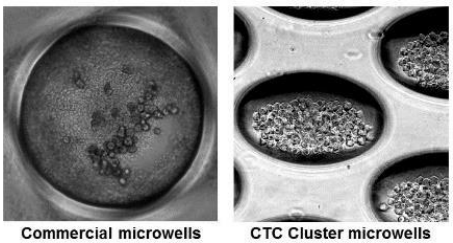

Figure 3: Importance of microwell array. Commercial cylindrical microwells form multiple irregular small clusters of 10–20 cells (centre). In contrast, cultures in tapered microwells of the microwell-based array consistently lead to the formation of a single large cluster at the centre of each microwell.

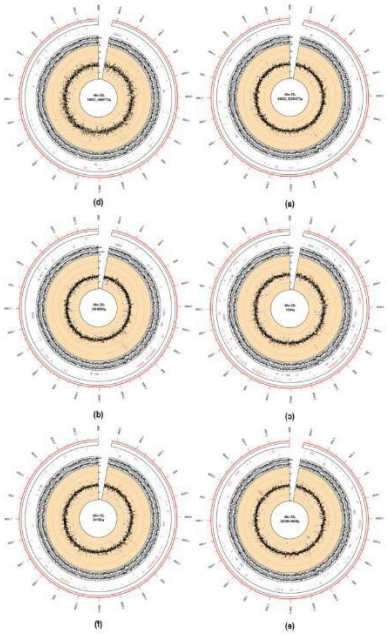

Figure 4 Transcriptome circles of Escherichia coli carrying MDR plasmids J53/pCTXM123_C0996 (a), J53/pCTXM64_C0967 (b), J53/pHK01 (c), J53/pNDM-HK (d), J53/pNDM-HN380 (e), and J53/pJIE143 (f). The outermost circle (in red) shows the genome coordinates (in Mbp) of J53. The first inner circle denotes the gene names of transconjugants, highlighting those with a log2-fold change greater than one (upregulated in red and downregulated in blue) compared with J53, as well as the genomic locations of these genes. The second inner circle demonstrates the gene expression of J53 and MDR plasmid transconjugants in TPM (transcripts per million) on the log2 scale. The third inner circle depicts the log2-fold change in gene expression of MDR plasmid transconjugants compared with J53. In this circle, the red and blue dots represent upregulated and downregulated genes (log2-fold change greater than one), respectively, while the other genes are colored in black.

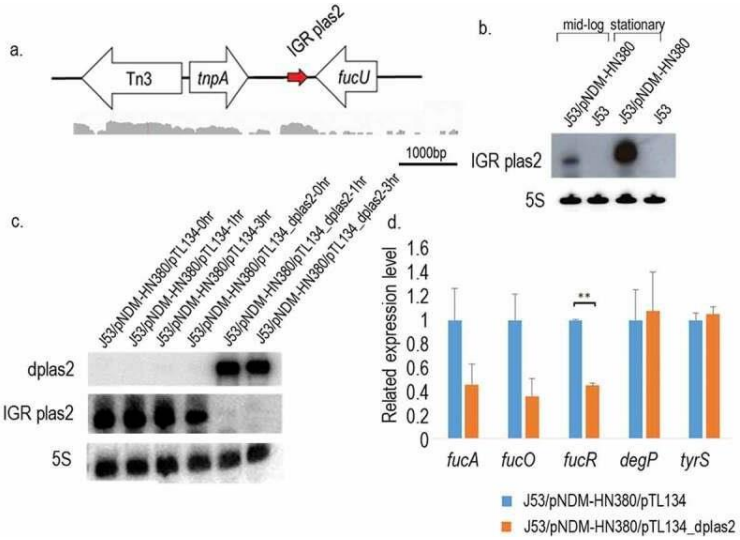

Figure 5. sRNA IGR plas2 and its decoy dplas2. (a). The genetic locus of IGR plas2. The arrow depicted in red is IGR plas2. RNA-sequencing reads showed below were captured using the Integrative Genomics Viewer (IGV) software. (b). Northern blot of IGR plas2. The experiment was performed at both log and stationary phases. The 5 S rRNA was used as control. (c). Northern blot validating the expression of dplas2 and the degradation of IGR plas2. The level of IGR plas2 was measured using the Northern blot assay at time points of 0, 1 and 3 hours after induction of dplas2. 5 S rRNA was used as control. (d). qRT-PCR of IGR plas2 target genes. Relative mRNA levels of predicted target genes of IGR plas2 were measured after the knockdown of IGR plas2. fucA, fucO, and fucR are functional genes in the fuc operon. J53/pNDM-HN380/pTL134 were used as controls. IGR: Intergenic region. Bars indicate the standard deviation. ** p-value