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Review

Standardising RNA profiling based biomarker application in cancer—The need for robust control of technical variables

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ABSTRACT

Histopathology-based staging of colorectal cancer (CRC) has utility in assessing the prognosis of patient subtypes, but as yet cannot accurately predict individual patient's treatment response. Transcriptomics approaches, using array based or next generation sequencing (NGS) platforms, of formalin fixed paraffin embedded tissue can be harnessed to develop multi-gene biomarkers for predicting both prognosis and treatment response, leading to stratification of treatment. While transcriptomics can shape future biomarker development, currently < 1% of published biomarkers become clinically validated tests, often due to poor study design or lack of independent validation. In this review of a large number of CRC transcriptional studies, we identify recurrent sources of technical variability that encompass collection, preservation and storage of malignant tissue, nucleic acid extraction, methods to quantitate RNA transcripts and data analysis pipelines. We propose a series of defined steps for removal of these confounding issues, to ultimately aid in the development of more robust clinical biomarkers.

1. Variability in colorectal cancer transcriptomic studies

Colorectal cancer (CRC) is the United Kingdom's second most common cause of cancer deaths [1]. Diagnostic staging of CRC relies on the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) staging system [2]. Classification into AJCC staging groups is based on extent of local tumour invasion, regional lymph node involvement and evidence of distant metastasis. While this staging system has intrinsic prognostic value, this initial categorisation is insufficient for predicting outcome following treatment [3]. Several clinical and histopathological markers have been reported to be 'prognosis predictors' for Stage II patients, by indicating benefit from adjuvant chemotherapy. These include extramural vascular invasion, grade 3/poor differentiation, serum carcinoembryonic antigen levels, T4 stage/perforation, perineural invasion, *CDX2*, obstructive tumours, mucinous tumours, tumour budding and microenvironmental factors such as immune cell infiltration [1,3–9].

Biomarker development aimed at guiding clinical decision-making for adjuvant therapy has been undermined by a lack of translation to clinical applicability, leading to a high attrition rate in effective cancer biomarkers. This is due in part to confounding issues such as inadequate study design, poorly selected clinical cohorts, technically inadequate assays, invalidated technology, inappropriate statistical analysis or lack of clinical relevance [10]. Thus, additional molecular-based biomarkers, informed by underlying CRC biology and the contributing role of the microenvironment, are required to stratify patients for biology-informed treatment.

Abbreviations: CRC, colorectal cancer; AJCC, American Joint Committee on Cancer; TNM, Tumour Node Metastasis; GEP, Gene expression profile; CRCSC, CRC Subtyping Consortium; CMS, consensus molecular subgroups; MSI, microsatellite instability; FFPE, formalin fixed paraffin embedding; S:CORT, Stratification in COloRecTal cancer; PAC, probably approximately correct; SOP, standard operating procedures; FF, fresh frozen; IVT, *in vitro* transcription; RIN, RNA integrity number; BBRB, Biorepositories and Biospecimen Research Branch; CDP, Cancer Diagnosis Program; TCGA, The Cancer Genome Atlas; ERCC, external RNA controls consortium; LDT, laboratory developed test; CLIA, Clinical Laboratory Improvement Amendments

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2. Comparison of single and multi-gene diagnostic testing: the EGFR paradigm

Molecular DNA-based biomarker testing, traditionally involving analysis of a single gene/mutation, have made important contributions in modern therapeutic decision-making in CRC, but may also have certain limitations. An example of the limitations associated with low throughput-gene testing is evident from the patient stratification approach used for cetuximab and panitumumab, monoclonal antibodies which target non-identical epitopes on the extracellular domain of *EGFR* [11]. Screening for mutations in *KRAS* exon 2 (codon 12/13) was initially employed to predict lack of response to anti-EGFR therapeutics, but recently studies postulate potential greater clinical utility in assessing additional mutations in *KRAS* exon 2, 3, and 4 and *NRAS* exons 2, 3, and 4, *PIK3CA* and *BRAF* mutations and *HER2* amplification [12–14]. Multi-gene assays, which encompass screening of at least *NRAS*, *KRAS*, *PIK3CA* and *BRAF* mutations, could enable better characterisation and ultimately more precise targeting of several druggable EGFR pathway components using therapeutic combinations to circumvent chemoresistance [15]. Transcriptional outlier analysis can be used to identify high expression of mRNA transcripts in individual samples, in comparison to the remainder of the cohort, which encode proteins that can be therapeutically targeted enabling individualised treatment options. Transcriptional outlier analysis of cetuximab resistant CRC cell lines, which were wild-type for KRAS, NRAS and BRAF has enabled the identification of tyrosine kinase that can be therapeutically targeted to overcome cetuximab resistance [16].

3. Requirement for a common approach to transcriptomic analysis of clinical specimens

In recent years, numerous efforts to precisely define the molecular landscape of CRC using transcriptional array-based studies have enabled classification of patients into 3–6 subgroups based on their underlying gene expression [17–22]. Recently, a CRC Subtyping Consortium (CRCSC) established a consensus molecular CRC classification comprising four subgroups (CMS1-CMS4) based on gene expression profiling (GEP) data from six key molecular taxonomy papers [23]. The CRCSC classifier demonstrated a robust performance across GEP platforms and sample collections, although the clinical value of prospective patient classification according to CMS subgroup for treatment selection remains to be tested. Importantly, while each study has identified prognostic biology associated with their identified subtypes, with the exception of the microsatellite instability (MSI) subtype (CMS1) and another subtype defined by high expression of mesenchymal genes (CMS4) [23] there are very few similarities between biological characteristics of the biomarker-driven classifiers that select these subgroups. On detailed examination, of the six classification signatures used in the CRCSC study, we identified only one gene, Quinolinate phosphoribosyltransferase (*QPRT*), present in all signatures [24] (Fig. 1). The protein encoded by the *QPRT* gene is involved in *de novo* NAD biosynthesis using quinolinic acid [25]. The lack of common genes in different molecular, predictive or prognostic classifiers is often due to a combination of (i) small training sets, (ii) cohorts being limited to one institution or (iii) samples from patients with differing percentages of AJCC stages being used in signature generation, resulting in low reproducibility in independent datasets [26,27].

The lack of commonality that we have highlighted between gene expression signatures may potentially be one of the reasons why multi-gene biomarker development rarely translates to a validated clinical test. Therefore, there is a requirement to examine the multi-gene biomarker development pathway to identify study design issues or technical variability that compromise robust gene expression signatures development for stratification of patients by prognosis or response to treatment. The multi-step biomarker validation pathway involves assessment of; (1) availability and quality of clinical specimens, (2) transcriptomic assay performance characteristics, (3) data pre-processing algorithms, (4) mathematical predictor model development (5) and assessment of its performance, (6) clinical interpretation of the test result, (7) clinical trial design, (8) ethical, legal, and regulatory issues [28].

In this review, we focus on the early steps of this biomarker validation pathway and examine in detail the level of scrutiny an investigator, looking to initiate a transcriptomic profiling study, should achieve to negate the introduction of study and/or technical variability. To help the reader understand the complexity involved in transcriptome profiling studies, we have highlighted the different methods published by independent CRC transcriptome profiling studies [29–63]. In the online Supplementary Table 1, we provide a summary of 57 transcriptome profiling studies and detail relevant information regarding the study cohorts such as small cohort sizes, different proportions of patients with stages 1–4 and number of study sites. We also highlighted that groups have differences in the level of tumour content they found acceptable for the study and the way in which the tissue was preserved. There are two common tissue preservation methods currently used for GEP studies; fresh frozen (FF) and formalin fixed paraffin embedding (FFPE), with FFPE almost universally used, due to retention of morphological features. FFPE is a well-established preservation method for histopathological assessment but results in extensive RNA fragmentation and cross-linking, impacting on high quality GEP. It is advantageous if GEP studies can address the RNA fragmentation and cross-linking issues, thus enabling FFPE samples to be utilised, as it is estimated that a billion FFPE samples are archived in hospitals and tissue banks, often with substantial clinical follow up [64].

Aside from variables related to collection, preservation and storage of the tissue we noted that investigators often used different clinical endpoints to define good and poor prognosis which could potentially confound validating gene expression signatures on independent cohorts. To complicate matters, investigators have employed different RNA extraction protocols and microarray platforms from a number of different vendors (Affymetrix, Illumina, Agilent, Almac) to perform the quantitation of the RNA transcripts and the probe sets utilised by different array manufacturers can differ in size and also the target region they anneal to. Therefore, it is not uncommon to find in some validation studies that expression data derived from specific probe sets is often omitted when comparing a gene signature established using one microarray platform with gene expression data derived from a different vendor's platform. Of note, published studies utilised different pre-processing algorithms such as RMA, MAS 5.0, iterPLIER and other vendor specific packages which can affect the outcome of whether a gene is included or excluded in a multi-gene biomarker. We also detail the number of studies which do not conduct an independent validation of their developed gene expression signature. In Table 1A–C, we provide a synopsis of the online Supplementary data to highlight the technical variability that occurs within multi-gene signature based biomarker CRC studies aimed at defining molecular taxonomy [17–22], patient prognosis [6,13,65–73] or treatment response [74–78]. Standardisation of these studies was commonly confounded by an incomplete control of pre-analytical variables, variable performance on different high-throughput technologies, use of an assortment of bioinformatics curation approaches. Additionally, the resulting multi-gene signatures were often not subjected to validation on an independent cohort of patient samples. While the studies detailed in Table 1A–C primarily focus on array-based studies, several reports in the last 3–4 years have begun to describe the quantitation of RNA transcripts using a next generation sequencing approach, (RNA-seq), on FF resected tissue from CRC patients [79–82].

This review identifies sources of technical variability in the GEP array-based biomarker development pipeline using FFPE CRC tumours. We categorise pre-analytical and analytical variables into nine distinct sections which can confound development and validation of published molecular signatures, to aid in the standardisation of the pipeline. This