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Predictive response biomarkers in rectal cancer neoadjuvant treatment

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1. ABSTRACT

Locally advanced rectal cancer (RC) treatment is a challenge, because RC has a high rate of local recurrence. To date preoperative chemoradiotherapy (pCRT) is widely accepted as standard protocol of care for middle-low RC, but complete tumour response rate ranges from 4 to 44% and 5-year local recurrence rate is 6%. Better understanding of molecular biology and carcinogenesis pathways could be used both for pre-neoplastic lesions and locally recurrence diagnosis, and for tumour response prediction to therapy. Circulating molecules, gene expression and protein signature are promising sources to biomarker discovery. Several studies have evaluated potential predictors of response and recently, cell-free Nucleic Acid levels have been associated to tumour response to neoadjuvant therapies. Alternative method is the serum or plasma proteome and peptidome analysis. It may be ideally suited for its minimal invasiveness and it can be repeated at multiple time points throughout the treatment in contrast to tissue-based methods which still remain the most reliable and specific approach. Many studies have analyzed preoperative rectal tissue prognostic factor, but data are controversial or not confirmed.

2. INTRODUCTION

Surgery is the primary treatment for rectal cancer (RC). In locally advanced stages of the disease, surgery is usually supported by radiation or a combined therapy to reduce risk of local recurrence (1-5). Preoperative chemoradiotherapy (pCRT) is particularly attractive for the following reasons: 1) *a priori* not curatively resectable tumours can be downsized to achieve the tumour cell-free surgical margins (R0 resection); 2) preoperative treatment reduce tumour burden and increase the possibility for preservative surgery; and 3) skip postoperative clinical complications precluding subsequent adjuvant chemoradiotherapy. The chemotherapeutic drug commonly used in RC treatment is *5-fluorouracil (5-FU*), which arrests DNA synthesis and causes interruption of the duplication of the cell. Current standard treatment includes the administration of ionizing radiation for 45-50.4 Gy in 25-28 fractions associated with 5-FU. After pCRT the complete pathological response is approximately 20%, whereas in 20 to 40% of patients the response is poor or absent (6, 7). This poses a considerable clinical dilemma because patients with *a priori* resistant tumour could spare radiations or DNA-damaging exposure treatments with

substantial adverse effects and so undergo to surgery without delays. In this frame, the identification of predictive markers of cancer response to pCRT is surely of significant clinical relevance. Several studies have been performed in this way, but findings are still unclear and controversial (8, 9): patient selection, sample size, study design, treatment modality and tumour response definitions are the major discrepancies and the only accepted marker is Carcinoembryonic Antigen (CEA) (10, 11).

Here, we summarized some approaches used for tumour response prediction through the study of genomics, gene expression (by microarray technology) and the description of the circulating peptidome (by mass spectrometry analysis) and cell-free nucleic acids. In our opinion, a systemic approach aiming at integrating different data (e.g. gene expression data with proteins/peptides biomarkers and molecular/histological determinants of cancer staging and progression) has the potential to provide high content information about patients' diagnosis and prognosis.

3. TISSUE-BASED BIOMARKERS ANALYSIS

Gene expression signatures found in different study has limited overlap of genes and results of testing published on different tumour cohorts is useless. Results are conflicting and still remain inconclusive both for technical and clinical differences. Collection of tumour biopsy before the treatment, usually carried out during colonoscopy or rectal exploration, rarely give enough tumour material. The lack of standardized clinical management rules, differences on the sample manipulation (collection, storage and processing) and differences in result evaluation make difficult a comparison between data. Furthermore, several recent publications have provided evidence of tumour microenvironment involvement in modulating tumour response to chemoradiotherapy. This is due to molecular factors expressed by neighbouring cells involved in tumour resistance (chemotactic molecules, growth factors, death factors) and regulating immune system cells recruitment. Moreover, irradiated cells can induce mutagenic response in neighbouring cells not directly traversed by particle radiation by gap junctions (12-15).

3.1 DNA alterations: polymorphisms

To date pharmacogenetics plays an important role in cancer chemotherapy and prognosis can be explained with genetic background or individual influence (16).

In the term of associations between cancer prognosis and genetic markers, DNA alterations are still regarded as great challenges in the field of tumour chemotherapy sensitivity. A widely used antineoplastic agent, oxaliplatin, acts as inhibitor of cell replication by DNA damage or macromolecular adducts formation. In colorectal cancer (CRC), the success of oxaliplatin chemotherapy is remarkable whether in advanced colorectal cancer (aCRC) or metastatic colorectal cancer (mCRC) (17, 18). However, drug resistance related to genetic variations is one of the main causes of treatment failure and the evaluation of pharmacogenetic markers may benefit cancer patients to individual prognosis (18-21).

In order to explore the influence of genetic variation by oxaliplatin-based chemotherapy *XRCC1* (Arg399Gln) and *GSTP1* (Ile105Val) polymorphisms have been widely studied on prognosis of colorectal cancer, but those conclusions were inconsistent each other (21-24). The *XRCC1* Arg399Gln polymorphism has been considered to increase chemotherapeutical sensitivity, but reducing the function of DNA repair, it also leads to increase DNA damage and mutation induction (25). Indeed cells with a switch from arginine to glutamine, such as the Arg/Gln or Gln/Gln, show negative effect on the DNA repair activity. Theoretically, these cells would have larger amounts of DNA damage, and therapeutic effect of oxaliplatin-based chemotherapy should be turn better (26). Additionally, a proper tumour description (e.g. tumour classification and stages) may be another factor accounting for those inconsistent results, both for aCRC and mCRC (20).

However, results showed tumour response rate is significantly lower in patients which carried Arg/Gln+Gln/Gln than Arg/Arg polymorphisms in *XRCC1*. For these patients, a stable or progressive disease was regarded as non-responsive event, which is opposite to the previous study (26) but is consistent with others (24, 27). Other genetic variations of *XRCC1* may be also attributable to the prognosis, such as the linkage disequilibrium with other genes with similar mechanisms. The polymorphism combination with each other could result in significant different effect or contribute to strengthen the *XRCC1* Arg399Gln polymorphism mechanism (25, 20). Platinum-based agents are commonly used in several solid tumours with successful (21). However, genetic variations influence the tolerance to drug-dependent DNA adducts, DNA repair protein complex function and drug metabolism that lead to negative effect on prognosis (18, 19, 25, 28).

3.2 Gene expression

Gene expression signatures by microarray technology may help to predict tumour response after pCRT. Recent studies have shown gene expression profiles of tumour cells discriminating responders and non-responders patients underwent neoadjuvant or adjuvant chemotherapy (29-32).

However, several papers have been focused on the evaluation of gene expression profiles on RC biopsies after neoadjuvant therapy, but some authors using different treatment protocols: one using radiotherapy alone and two added Cetuximab to conventional pCRT, instead in other three works the study design shows small number of cases to draw firm conclusions (33-38). Each study provided classifiers with high predictive accuracy but a little overlap is observed between the gene lists. They predict similar outcomes when the same tissue type is carefully compared and only a handful of identical matches are evident. For example, in Agostini *et al.* (submitted), Rimkus *et al.* (37) and Kim *et al.* (38) are similar studies, about enrolling of patients, treatment protocol and technology to investigate

response to pCRT in RC and no predictor gene is in common. Validation some common genes in previously published data highlights conflicting findings in studies on the same disease. Probably this occurs for various reasons such as: different technical methods of tissue preparation, different type of platform used or expression profiling technologies, different workplaces and different methods of data analysis (39).

Large-scale meta-analysis techniques remain the best way to identify gene sets associated with response to therapy and disease free survival by the integration of results of retrospective analysis and the *de-novo* analysis from raw data.

Recent advances in computational algorithms and computing power allows the analysis, management and use of large sets of genomic and proteomic information. Once statistically analyzed, results obtained might indicate treatment selection and predict patient outcome. In order to progress in this field, a deeper confidence in these classifiers must be established through repeated validations.

3.3 miRNA analysis

Mature miRNAs can interfere with protein expression in two ways: 1) in association with RISC (RNA-induced silencing complex) targets and cleaves mRNA, 2) or translational inhibition thought a imperfect complementarity sequence-dependent process, but the mechanism is still unknown (40). Excellent reviews describing the molecular biology of miRNAs have been published (41, 42). Here, we shall just summarize a few essential elements of miRNA involvement in tumour progression, treatment and outcome. Recently, the role of miRNA in drug resistance/sensitivity was realized.

In 2006, Nakajima *et al.* observed the expression level of *miR-200c* was significantly over-expressed in their colorectal tumour samples compared to the normal corresponding ones (43). *Let-7g*, which is known to target more than 200 mRNAs (including genes such as: *RAS*, *cyclin D*, *c-myc* and *E2F* transcriptional factors family), was over-expressed in tumour samples and was significantly associated with chemosensitivity to S-1-based therapy. Also, the expression of *miR-181b* (which is probably target mRNAs encoding genes such as *cytochrome c, ECIP-1, MAPPKKK1, TEM6, E2F5, GATA6, PP2B* and *eIF5A*) was strongly associated with patients' response to S-1 drug, but it is not significant for patient survival.

Rossi and colleagues demonstrated 5-FU can significantly change the expression levels of miRNAs in human colon carcinoma cell lines (HT-29 and HCT-116) (44). Quantitative Real-Time PCR revealed that 5-FU up-regulates 19 miRNAs, like *miR-133a*, whose targets are pro-apoptotic proteins (Bax and K-Ras), *miR-147* and *miR-27b*, and down-regulates 3 miRNAs like *miR-200b* and *miR-210,* which were associated to tumour cell proliferation inhibition and increase target cell apoptosis. A potential target gene of *miR-200b* is the Tyrosine- protein phosphatase non-receptor type 12 (PTPN12), which can bind dephosphorylated and inactivated products of oncogenes such as *c-Abl*, *Src* or *Ras*.

In another work, Svoboda *et al.* evaluated miRNAs expression in tumour biopsies from patients with RC before and two weeks after starting preoperative capecitabine chemoradiotherapy (45). They observed post-therapy increase levels of *miR-125b* and *miR-137*: *miR-125b* up-regulation seems down-regulate the insulin-like growth factor 1 receptor (IGFR-1), as well as the vascular endothelial growth factor (VEGF) and its receptor (VEGFR), thus suppress tumour growth and angiogenesis through insulin/insulin-like growth factor pathway, while *miR-137* up-regulation could be important to maintain tumour state.

4. BLOOD-BASED BIOMARKERS ANALYSIS

The dynamic nature of circulatory system and its constituents reflect physiological or several pathological states and the easiness of sampling procedures are a logical choice like source for biomarker discovery. DNA, mRNA and miRNA (cfNA, cell-free Nucleic Acid) are released in the blood of cancer patients. Changes in the levels of circulating nucleic acids have been associated with tumour burden and malignant progression. In the past decade, a wealth of information on the possible use of circulating nucleic acids for screening, prognosis and monitoring the anticancer therapies efficacy has emerged. cfDNA, cfmRNA and cfmiRNA might be excellent blood cancer biomarkers, as they may be more informative, specific and accurate than usual protein biomarkers.

4.1 cfDNA

Physiological events leading to cfNA increase during cancer development and progression are still not well understood. However, analysis of circulating DNA allows the detection of tumour-related genetic and epigenetic alterations during development and progression of cancer. The presence of nucleic acids into bloodstream is thought to be related to the apoptosis and necrosis of cancer cells in the tumour microenvironment. Necrotic and apoptotic cells are usually phagocytosed by macrophages or other scavenger cells and digested DNA is released into tissutal environment (46). It has been estimated for a tumour that weighs 100g, which corresponds to 3 × 10^10 tumour cells, up to 3.3% of tumour DNA may enter in the blood every day (47). On average, the DNA size range between small fragments of 70 to 200bp and large fragments of approximately 21 kb (48). Secretion has also been suggested as a potential source of cfDNA (49, 50). Finally, tumour cells circulating in the bloodstream and micro-metastatic deposits present at distant sites, such as the bone marrow and liver, can also contribute to the release of cfNA (51, 52).

Although the major end-point of some studies was focussed on the role of cancer-related circulating cfDNA in early diagnosis, a most interesting challenge is the evaluation of its role in tumour response to pCRT in RC. Agostini *et al.* were confirmed significantly lower levels of circulating cfDNA in patients having relevant

Table 1. Circulating cfmiRNA expression related to response to chemotherapy

miRNA	Cancer type	Body fluid source	Patients (n)	Received therapy	Therapy-related significant associated end points	Ref.
miR-21	Prostate (hormone refractory)	Serum	10	Docetaxel and prednisone	Biochemical response (PSA)	(72)
miR-21	Metastatic NSCLC	Plasma	35	Cisplatin- or carboplatin-based chemotherapy	Radiologic tumour response	(73)
miR-210	Breast	Plasma	29	Neoadjuvant paclitaxel followed by FEC plus trastuzumab	Pathological tumour response	(74)
miR-375 miR-184 miR-1299 miR-196a miR-381 miR-410 miR-1246	Breast	Serum	23	Neoadjuvant doxorubicin and cyclophosphamide followed by carboplatin and nab-paclitaxel plus trastuzumab	Pathological tumour response	(75)
miR-125b	Breast	Serum	56	Adjuvant FEC or Docetaxel endocrine treatment according to hormonal receptor status	Radiologic tumour response	(76)

Abbreviations: FEC, 5-fluorouracil, epirubicin and cyclophosphamide; NSCLC, Non-small cell lung carcinoma; PSA, Prostate-specific antigen.

tumour regression after neoadjuvant therapy than non-responder patients to pCRT (53). In fact, while the baseline levels of circulating cfDNA did not predict tumour regression, post-pCRT cfDNA integrity index (long/short fragments ratio) has been negatively associated with therapeutic response. These findings confirm plasma long fragments represent cfDNA relative to the tumour, while short fragments represent a heterogeneous source of cfDNA. Moreover, cfDNA possess clinical significance when considered as a dynamic process: the variations in the cfDNA long fragments and the cfDNA integrity index during the treatment seem to have superior clinical value than a single pre- or post-pCRT assessment (54-56). Indeed, considering each patient, cfDNA quantity showed an elevated variability, indicating that a clinical use of this marker will require further studies and refinements. The reasons of this variability could be due to different factors affecting cfDNA release: apoptosis; tumour necrosis; T-cell and mitochondrial origin; low activity of DNase I and II; spontaneous and active release of DNA by proliferating cancer cells and activated lymphocytes (57-62). Cell turnover, immunological response, aggressiveness of the disease and apoptosis induced by the pCRT are the next task in future studies (63, 64).

Although cfDNA basal levels actually can not be used as markers of the response, significant differences between responder and non-responder patients are found during treatment (e.g., after the first 2-3 weeks of pCRT). These observations, with the association of clinical instrumental investigations (like transrectal ultrasound, pelvic computed tomography scan or magnetic resonance imaging, abdominal/chest computed tomography and CEA test),will permit a modification of the therapy in real-time. For non-responder patients, a useless pCRT treatment could be stopped and they may undergo to surgery.

In conclusion, circulating cfDNA levels and its integrity index have great potential to be used as prognostic markers of rectal cancer.

4.2 cfmRNA and hTERT

Levels of cfmRNA have been assessed in plasma of patients with different malignancies, including colorectal cancer (65, 66). Because high levels of cfmRNA have also been found in benign conditions, such as the placenta-derived cfmRNA in maternal circulation and other non-malignant conditions, its specificity should be considered with caution (67, 68). Conversely, hTERT expression is inappropriately activated in the most tumours and, because absent in non-neoplastic somatic tissues, its detections into bloodstream may be considered as a specific neoplastic marker.

On the other hand, in Pucciarelli *et al.* study, cfmRNA and hTERT variation have been correlated to response to pCRT (69). Plasma levels significantly decrease in patients with response to therapy, while remaining unchanged -or even increased- in non-responder patients. Since cfmRNA is released by different mechanisms (cell necrosis of large and advanced tumors, cell apoptosis or spontaneous and active release) their higher levels in non-responder patients could suggest a more active necrosis or a major tumour extension than responder patients (65, 70). Because of hTERT was found to be absent in plasma of healthy subjects, it was intriguing that hTERT was present in patients with a pathologic complete response. Circulating microscopic disease or low hTERT clearance from plasma are both possible explanations (66, 69). This can be further clarified by assessing hTERT levels at different time points after the completion of pCRT (i.e., several months after surgery).

Despite this study has limitations related to its retrospective nature, the relatively small number of patients and the non-uniform regimen of treatment, results prompted seem to be a good background to verify the predictive value of both cfRNA and hTERT in rectal cancer.

4.3 cfmiRNA

Recent findings demonstrate that blood contains stably expressed tumour-specific miRNAs (71). Serum or plasma miRNAs are easily accessible and are stable even under severe condition changes, such as pH and temperature variations. This represent an ideal staring point for biomarker assessment, and a lot of studies have been

focused on their potential role as diagnostic/prognostic biomarkers for cancer detection and monitoring (Table 1).

In the last years, a few studies have pointed out an encouraging correlation between circulating miRNA expression levels and response to a specific anticancer treatment. A first study has been performed on a small set of hormone-refractory prostate cancer patients and specific serum levels of *miR-21* were measured in all docetaxel-treated patients (72). Circulating *miR-21* was over-expressed in patients resistant to chemotherapy and resulted predictive of the response to docetaxel-treatment and to indicate the transformation to hormone refractory disease.

In a similar way, authors highlighted a predictive role of circulating *miR-21* expression level in patients affected by NSCLC and treated with platinum derivative-based chemotherapy (73). More recent publications have discussed the predictive role of circulating miRNAs in breast cancer. The study by Jung and colleagues considered the baseline expression of 4 candidate miRNAs (*miR-210*, *miR-21*, *miR-29a* and *miR-126*) in breast cancer plasma samples and only *miR-210* appeared to be significantly over-expressed in patients resistant to the trastuzumab-based treatment (74).

In the study by Wu *et al.* small ncRNAs (not coding RNA) miRNA extracted from patients' serum were analyzed by next-generation deep sequencing to detect differential expression levels between different classes of breast cancer patients (75). The relationship between tumour response to neoadjuvant trastuzumab-based treatment and miRNA levels was investigated in 23 patients. A seven miRNA signature was associated with pathological complete response (*miR-375*, *miR-184*, *miR-1299*, *miR-196a*, *miR-381*, *miR-410* and *miR-1246*). Specific panel of other miRNAs (*miR-10b*, *miR-34a*, *miR-125b* and *miR-155*) have been evaluated as predictive of adjuvant treatment outcome in breast cancer patients and only high expression levels of circulating *miR-125b* were associated (76). Findings were further tested on primary cancer cells isolated from pre-treatment biopsies and they confirmed *miR-125b* over-expression in primary breast cancer cells with poor tumour response.

Although a huge literature is present, more accurate and extensive studies are needed to appreciate the real value of circulating miRNA expression as a predictive tool in personalized cancer treatment. In particular, some clarifications are necessary: although short ncRNA have been detected in the extracellular culture medium of mammalian cells *in vitro*, the release mechanism from tumour cells is still unclear. Extracellular miRNAs seem to be transported by lipoprotein complexes originating from endocytosis of endosomal cellular membranes called micro-vesicles or exosomes, also containing mRNAs and proteins (77). This mechanism of extracellular transport would justify the stability of miRNA in circulating body fluids. However, some authors have recently demonstrated the most of circulating miRNAs are outside exosomes and their stability could be owing to complex formation with Ago proteins (78).

Therefore, the source of cfmiRNAs and the extraction methods could bias the results of studies and thus limit the searching field and ignoring the vast majority of the circulating miRNAs or focusing on non-tumour miRNAs. These controversial data highlight the necessity of more studies to establish standardized and robust methods for detecting circulating tumour cfmiRNA.

5. CIRCULATING PROTEINS/PEPTIDES

Circulating proteins reflect the complexity of molecular processes involved in cancer, and their identification and characterization in the field of proteomic studies (79). To date, proteome profiling for identification of therapy-related changes, which could be used for monitoring progression, efficacy and toxicity of the treatment in rectal cancer, has been scarcely investigated.

In a study, Smith *el al.* performed a SELDI-based serum profiling in patients with RC undergoing pCRT at several time points during the therapy: before and after each serial radiation treatment (80). This study revealed specific features of proteomic profiles, discriminating patients with good and poor histological response to the therapy. In particular, a pattern of 14 differentially abundant proteins has been found to predict the ultimate pathologic response with 87.5% sensitivity and 80% specificity. If these results suggest that early proteins changes after cytotoxic therapy are clearly detectable, proteins identity was not clarified and needed further investigation. In a similar way, Helgason *et al.* found 2 proteins having changed serum level correlated with therapy response in CRC patients treated with oxaliplatin and capecitabine (81). Proteins have been tentatively identified as a probable fragment of hemoglobulin alpha-chain fragment (MW 2kDa) and the acute phase Apo A-I protein (MW 28kDa). If these proteins were found useful for therapy monitoring, data obtained were not conclusive regarding their predictive value and required further test.

Beside proteome, peptidome has been recently recognized as a novel source of biomarkers, which could improve diagnosis, prognosis and monitoring of various diseases including cancer (82). Peptidome is the sub-proteome fraction, including intact peptides or active peptides released form precursors under specific physiological conditions (e.g. immune response), or peptides originating from protein degradation pathways. Tumour microenvironment, through its aberrant processes of cell growth, cellular invasion, alteration of immune system function and angiogenesis generate a unique cascade of events that lead to specific protein fragmentation products (83). Moreover tumour-related peptides can be originating both from apoptosis and necrosis events of cancer cells in the tumour microenvironment, and from released proteases into the bloodstream. In literature, comparative analyses of circulating peptidome profiles in healthy subject and patients with different kinds of cancer have been performed, allowing the identification of peptide signatures that could be peculiar of pathology or tumour site (79, 84, 85).

Even if these studies are promising, a positive application in the detection of peptidome changes in pCRT treated rectal cancer patients is still lacking.

6. CONCLUSION

Predictive factors of tumour response in patients receiving neoadjuvant treatments are clinically relevant. Currently, although many molecular markers are studied as potential candidate predictors, none have been introduced in clinical practice. Many results were obtained by gene expression signatures to predict tumour response after pCRT. Different research approaches to identify sub-phenotypes of rectal cancer are the best opportunity to head the clinical research to individualized therapy. Despite all advances obtained, few studies have attempted to demonstrate the value in integrating genomic and proteomic information with the traditional biomarkers for providing a detailed assessment of clinical risk and improving prediction of response to therapy. The presented studies could significantly improve knowledge and application of gene expression, peptidome profiling and characterization and quantification of circulating cfNA, to a clinical predictive classifier of therapeutic response in rectal cancer. Novel interpretation ways we could go through integrating the various findings and leading to identify important molecular factors in the prediction of response to treatment.

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