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Clinical proteomics in oncology : a passionate dance between science and clinic

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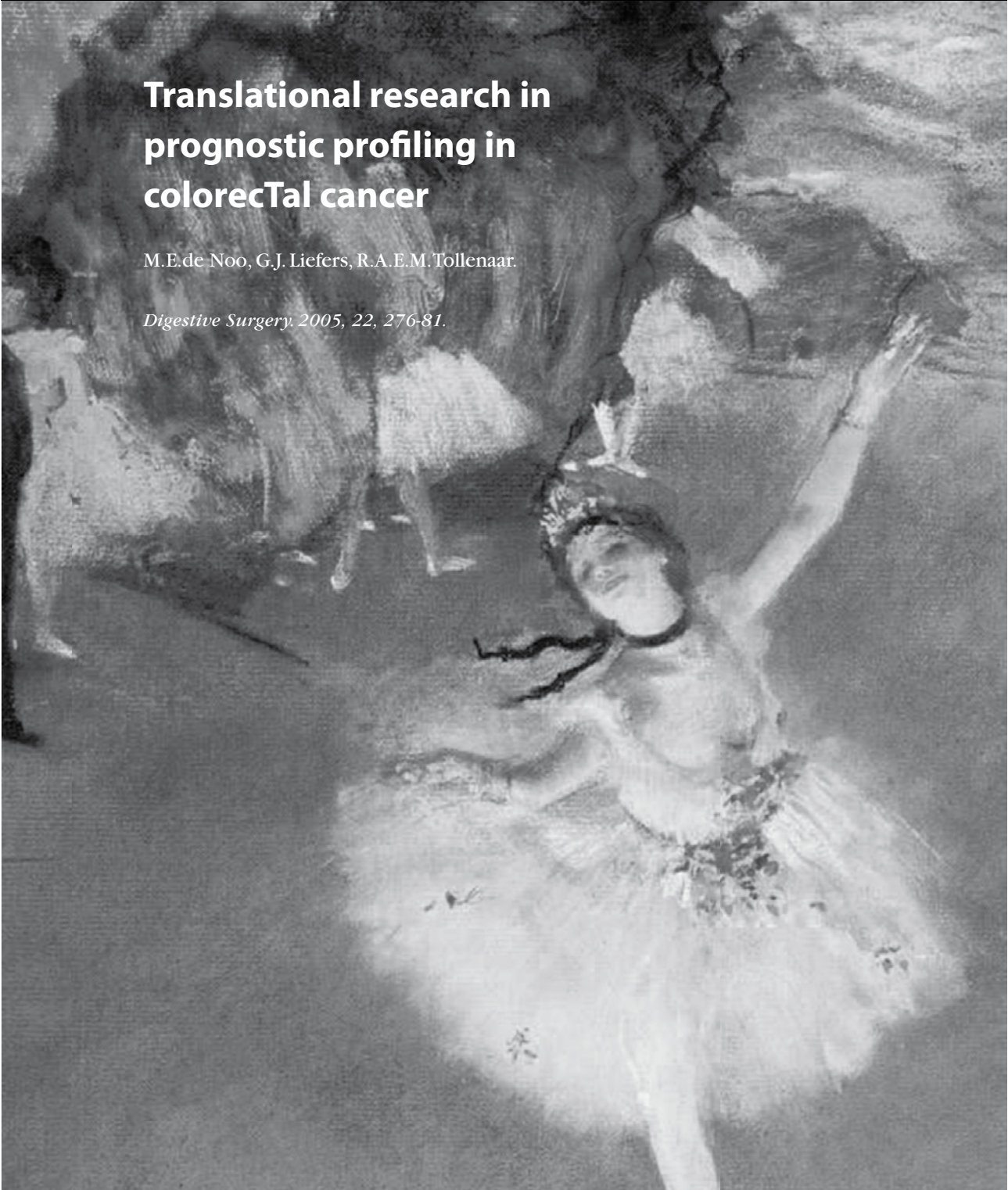
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Chapter 2

Translational research in prognostic profiling in colorecTal cancer

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ABSTRACT

There is a widening gap between basic research and clinical practice, particularly for colorectal cancer. In recent years, many have expressed concerns regarding the disconnection between the promises of basic science and the delivery of better individual health. In this paper we describe some of our research in serum proteomics, microarrays and minimal residual disease dedicated to this field and discuss some of the roadblocks ahead in translational research. We conclude that translational medicine should be a collective effort for the medical community as a whole with adequate financial support and sound, measurable outcome. Since extensive validation of the above mentioned research fields is necessary, adequate funding is required. This may require some adjustments in the current funding policy because it involves non-innovative studies. Furthermore, the pool of researchers/clinicians capable of performing translational research must be increased. Additionally, there should be an enhanced participation of patients in clinical trials and an optimization of the efficiency of these trials using validated surrogate markers. Only when these conditions are fulfilled will the 'post-genomic' era of biomedical research have unprecedented opportunities to innovate and improve therapy for cancer.

INTRODUCTION

There is a widening gap between basic research and clinical practice particularly for colorectal cancer. Over the last decades our molecular knowledge on the genesis of colorectal cancer has increased dramatically. Despite this increase our treatment of patients remains largely the same: ‘en-bloc’ removal of the primary tumour and draining lymph nodes when possible, staging according to standard Dukes’ or TNM classification systems and adjuvant treatment with cytotoxic drugs and/or radiation therapy. Despite mounting evidence of abundant heterogeneity of both clinical course of disease and responsiveness to therapy, ‘tailor-made’ medicine is an item in review papers and editorials instead of every-day-practice.

The paradigm for the translation of new information has been conceptualised by some as a highway. A ‘translational highway’ running from basic biomedical research to individualised patient care with improved health as a result (Figure 1). In recent years many have expressed concerns regarding the disconnection between the promise of basic science and the delivery of better health.[1] In a special communication for the JAMA, Donald Berwick addresses the problem of disseminating

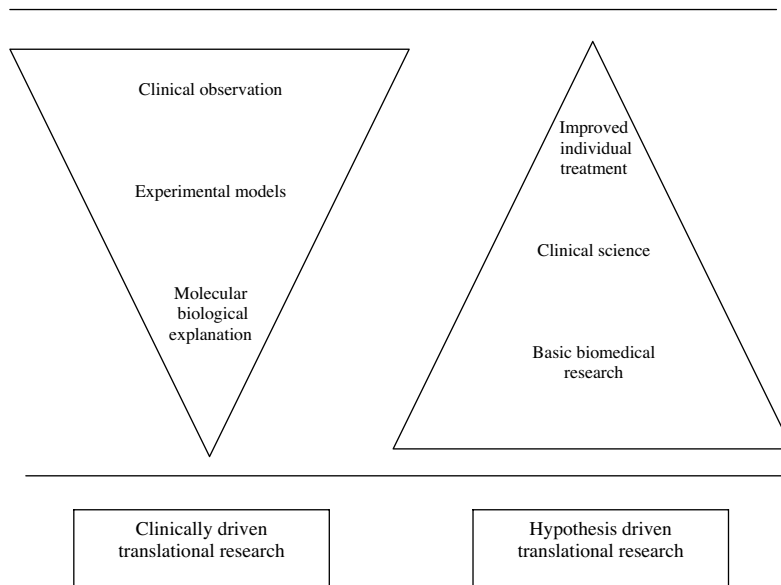


Figure 1. Current biomedical research places high priority on defining molecular mechanisms of disease with the ultimate aim of improving health of the individual patient (hypothesis driven). However, part of the failure to translate hypotheses derived from complex experimental models into improved patient care can be explained by the fact that many of these hypotheses do not translate to human pathology. It is therefore pivotal for successful translational medicine to promote research based on clinical observations and corresponding molecular biological explanations (clinically driven).

Table 1. *Factors associated with perceptions of an innovation***Perception of an innovation that influences the rate of spread**

Perceived benefit of the change
Compatibility with beliefs and needs of potential adopters
Complexity of the proposed innovation
Trialability (testing the change on a small scale)
Observability (watching others try the change first)

Adapted from: E.M. Rogers, *Diffusion of innovations*, 4th ed. New York, NY: Free Press 1995

innovations in health care as postulated by Rogers.[2] One of the obstacles he mentions, is that apart from lack of knowledge about the expected consequences of innovations or the perceived benefit, these innovations must resonate with currently felt needs and beliefs. Other factors associated with perceptions of an innovation are the complexity the proposed innovation, trial ability (testing the change on a small scale) and observability (watching others trying the change first), as shown in table 1. For colorectal cancer the ‘needs and beliefs’ are evident. First, the prognostic information from our standard classification system needs refinement. This is exemplified by the fact that despite of lack of evidence of residual disease in Dukes’ B colorectal cancer patients, 30% die of recurrent disease within five years. Second, there is a need for tools that allows us to predict or monitor therapy response to avoid unnecessary morbidity. And third there is a need for new molecular targets that allows the development of cancer specific drugs that lack the side effects of current cytotoxic chemotherapeutics.

In this paper we would like to describe some of our research dedicated to this field and discuss some of the roadblocks ahead.

PROTEOMICS

Cancer is often described as a genetic disease. A gene alone, however, is only potential information that must be put into a functional form. The DNA is transcribed into RNA before translation into protein, the manifestation of the genetic code. During the transformation of a healthy cell into a neoplastic cell, including alterations in expression, activity and localization and differential protein modification, changes occur in the protein level. Identifying and understanding these changes is the underlying theme in cancer proteomics.[3]

Proteomic pattern diagnostics is a recent and potentially revolutionary approach for early disease detection, prognostication, and monitoring in oncology. The use of proteomic technologies might benefit biomarker discovery and treatment modalities: serum protein profiling for early disease detection and molecular signal mapping to instigate pharmacoproteomic therapeutic interventions.[4]

Several studies have shown that biomarkers can be identified on the basis of the presence/absence of multiple low-molecular-weight serum proteins using mass spectrometry technologies such as SELDI-TOF and MALDI-TOF.[5-9] Patterns of these peptides can be correlated to biological events occurring in the entire organism and are likely to change in the presence of disease. In oncology new types of bioinformatic pattern recognition algorithms have been used to identify patterns of protein changes in order to discriminate cancer patients from healthy individuals.[10] Furthermore, different profiles may be associated with varying responses to therapeutics and other clinically relevant parameters and may also serve as prediction for treatment outcome. Although serum protein patterns showed high sensitivity and specificity as an early diagnostic tool in several studies, critical notes have been made on biological variation, pre-analytical conditions and analytical reproducibility of serum protein profiles that would make it difficult to differentiate a normal from a pathological and/or malignant status.[11] In addition, the reproducibility of serum protein profiles has been questioned, which however relates more to the bioinformatic analysis of the measured protein profiles than the capturing and measuring techniques itself.[12-14] Thus, if proteomics spectra are ultimately to be applied in a routine clinical setting, collection and processing of the data will need to be subject to stringent quality control procedures.[15]

In a recently submitted study we assessed the reproducibility of our MALDI-TOF protein profiling procedure after capture and elution of serum peptides with C8 magnetic beads. Corresponding to the logistical conditions in a routine clinical setting, the effects of sample handling and storage, and also individual factors on the serum protein profiles were analysed. The reproducibility of the used capturing technique with C8 magnetic beads and MALDI-TOF analysis is acceptable and satisfactory for large discriminating studies. The time of blood collection and the number of freeze-and-thaw cycles had no influence on serum protein profiles. However, sample handling prior to serum centrifugation did have considerable effect on serum protein profiles. All together, we have shown in this study that effects of handling and storage procedures on serum protein profiles lie within acceptable limits. To prevent bias in classification studies we stress the importance of a standardised collection of all blood samples, from the point of sample handling and storage until freezing the samples. Although the importance of homogeneity and uniformity within sample groups must be stressed, variation of such factors cannot totally be

excluded in a clinical setting. The most important issues for discriminating studies at this moment are a standardised and well-documented sample collection and a thorough study design. Further research for the statistical data-analysis is needed. Due to the lack of discriminating profiles, serum protein profiling is not ready for introduction in a routine clinical setting. Nevertheless, based on the present data and these of Villanueva et al. [16], we feel that the methodology can be standardised to a level which allows application as a diagnostic and prognostic tool. Therefore, we are now in the process of carrying out a study to determine whether serum protein profiles can differentiate colorectal cancer patients from individuals with benign bowel disorders and healthy subjects. Further, identification and functional analysis of these discriminating proteins will render new insights on tumour development and environmental responsiveness.

MICROARRAYS (PROGNOSTIC FACTORS)

Over the last decades, numerous molecular factors with prognostic and predictive value have been described. Specifically loss of heterozygosity (LOH) of chromosome 18q and microsatellite instability (MSI) have been repeatedly implicated both as prognosticators as well as predictive for 5-fluorouracil based chemotherapy.[17] Despite multiple studies with large number of patients and unequivocal outcome data these markers have not yet found their way into routine treatment planning for patients with colorectal cancer. One of the reasons for this may be that the observed differences are studied retrospectively, which diminishes the expected benefit of using these markers in clinical decision making. Furthermore, with respect to the triability, it takes a tremendous amount of work for potential adopters to prospectively validate these markers. Also, the use of a single marker disregards the biological complexity of tumour development. New techniques, such as cDNA microarray analysis enable the parallel monitoring of expression levels of thousands of genes. Current cDNA microarray protocols are based on the Southern blot technique in which labelled nucleic acid molecules are hybridised to complementary nuclear acid molecules attached to a solid surface such as glass. Technical innovations such as miniaturization and fluorescence-based detection greatly enhance the throughput. A microarray consists of thousands of small spots of multiple copies of amplified cDNA spotted on a glass microscopic slide. Each spot represents a unique sequence from a named gene or expressed sequence tag (EST) and one slide can hold up to 10,000 probes. As a target for analysis, total RNA or mRNA from two cell populations is used (e.g. cell lines, clinical samples and animal models). Fluorescent marker dyes such as Cy3 and Cy5 are incorporated into target cDNA. The labelled cDNA from the

two cell populations of interest are mixed with a labelled control sample and hybridised to the probes on the glass slides. The array is scanned using confocal laser microscopy. After excitation and emission of fluorescence, signals can be measured and displayed. This results in a matrix of thousands of green, red and yellow spots. When, for example, a gene is equally expressed in test and control samples, both the red and green fluorescent signals will be equally strong and will be visualised as a yellow dot. Consequently, in the case of differential expression, the red to green ratio will shift. Following hybridisation and scanning, large amounts of data are available for processing. A variety of software tools are available which can help to measure fluorescent signal ratios, exclude artefacts and normalize data.

In a small, unpublished series of rectal cancer patients we have tested the hypothesis that microarray analysis could distinguish between patients with and without liver metastases. In collaboration with the Institute of Medical Sciences, University of Tokyo, Japan, we analysed tumour RNA from 20 rectal cancer patients; 12 patients with liver metastases and 8 patients without. RNA was extracted from fresh frozen tissue samples using laser capture micro dissection (LCM). After amplification and labelling, probes were hybridised to a microarray consisting of 9,216 genes. After scanning, the differential expression ratio for each gene was determined.

Data were analysed according to the 'leave-one-out' methodology as described. The resulting set of 30 genes could correctly predict the presence of liver metastases in 10 out of 12 patients. These data are currently being validated in a larger series. However these preliminary data show that, as in many other tumours, cDNA microarrays are promising new tools for the prognostication of patients with colorectal cancer.

For the translation of these experimental techniques into standard care, some of the roadblocks ahead can be easily envisioned. First the proposed superiority over our standard classification system must be (repeatedly) demonstrated in large groups of patients. To achieve this, tissue banks with fresh frozen tissues and serum must be established for validation studies. With adequate funding, these tissues can be collected from patients who are randomised in clinical trials and made available to the research community. International initiatives from the NCI and EORTC underline this view.

Secondly, the introduction and acceptance of prognostic gene sets would be more anticipated when experiments show a causal role of each of the genes in the clinical course of the disease. Microarray data are therefore by no means endpoints. Rather, they are hypothesis driven starting points for the development of new therapeutic strategies.

MINIMAL RESIDUAL DISEASE

Detection of metastatic cells by molecular techniques has been reported to increase the sensitivity over standard pathological examination. Metastatic cells can be found in histopathological negative lymph nodes, bone marrow and blood of colorectal cancer patients. Many of the published papers indicate a poor prognosis in patients with molecular detected metastases in all of the mentioned sites. Despite this, molecular techniques are not routinely used in the staging of patients.

The prognosis for colorectal cancer patients whose lymph nodes are not involved (stage II) is good. Five-year survival rates approximate 70%. In the Netherlands, adjuvant chemotherapy, therefore, is not considered standard care. Our group studied 26 stage II patients to detect micro metastases in lymph nodes by reverse-transcriptase-PCR of carcinoembryonic antigen (CEA) mRNA in microscopic negative lymph nodes. Overall, micro metastases could be detected in one or more lymph nodes from 14 patients (54%). These patients fared significantly worse than the patients without micrometastases. In this study, survival dropped from 75% to 36% based on the presence of micrometastatic disease. When only cancer-related deaths were considered, survival dropped from 91% to 50% respectively. The relative risk for cancer related death associated with the presence of micrometastases was 11.7 (95% C.I.: 1.2-106.9; $P=0.03$).[18]

This study is one of the first to relate micrometastatic disease to patient outcome and provides a rationale for the selection of patients who might benefit from adjuvant therapy. Since our publication others have confirmed these findings but there has been no massive introduction of these techniques into daily practice. The reason for this is that the pivotal question whether the prognosis of patients 'upstaged' by molecular techniques improves after adjuvant treatment remains unanswered. The perceived benefit for this innovation therefore may be low and is subject of ongoing investigation by our group and others. A second reason for the lack of adoption of these techniques is that they are complex and time consuming. Sentinel node (SN) biopsy has been introduced to minimize the extent of surgery and to enable assessment of minimal residual disease (MRD) without compromising accurate staging or survival.[19] For colorectal cancer the SN concept could be used to limit the number of nodes amenable for detailed molecular analysis. We are currently in the process of evaluation of micrometastases in sentinel nodes from colorectal cancer patients.

Another area of research is MRD detection in bone marrow. Viable cancer cells can be found in bone marrow from 20-40% of patients with colorectal cancer. This phenomenon correlates with an adverse prognosis. We have tested different methods for MRD detection, including automated microscopy and RT-PCR and preliminary results indicate prognostic relevance of these tests for different stages of colorectal cancer.

[20] Not all cancer cells that can be found in bone marrow are clinically relevant since they are present even in patients that never relapse. Experimental studies in breast cancer show that tumours consist of heterogeneous populations of cells with distinct tumorigenic potential.[21;22]

Minimal residual disease may arise from tumorigenic or non-tumorigenic cancer cells. Only when tumorigenic cancer cells metastasize, clinically relevant metastasis will occur.[23] Support for this theory comes from observations that disseminated minimal residual cancer cells from patients with and without overt distant metastasis are genotypically different.[24] Therefore the development of diagnostic tools that allow for the prospective identification of tumorigenic minimal residual cells may have therapeutic significance for patients with solid tumours. This will be one of the research goals for our group in the coming years.

CONCLUSION

In the 'post-genomic era' of biomedical research there are unprecedented opportunities to innovate and improve therapy for cancer. These opportunities are limited by today's clinical infrastructure. Efforts to validate and implement novel therapies are characterised by lack of funding and fragmentation. For a successful translation of novel biomedical discoveries to improved, individual health there are several issues to be addressed. First of all, translational medicine should be a collective effort for the medical community as a whole with adequate financial support and sound, measurable outcome. As extensive validation of the above mentioned research fields is necessary, adequate funding is required. This may require some adjustments in the current funding policy as it involves non-innovative studies. Secondly, the pool of researchers/clinicians capable of performing translational research must be increased. Thirdly, there must be an enhanced participation of patients in clinical trials and we have to optimize the efficiency of these trials using validated surrogate markers. Especially when we move towards 'tailor-made' medicine, evidence from large randomised trials (with inherently large groups of uniformly treated patients) will be more difficult to obtain. Current clinical trials must be appended with basic biomedical science studies, with collection of tissues for retrospective analysis. Last, we have to deal with regulatory and cultural aspects of the implementation of health innovations.

For the coming years it is the goal of our group to integrate three lines of research; MRD detection, cDNA microarray analysis and proteomics (Figure 2). We believe that integrating these techniques will improve the detection and staging of colorectal cancer and allow more precise prediction and monitoring therapy responses of individual patients.

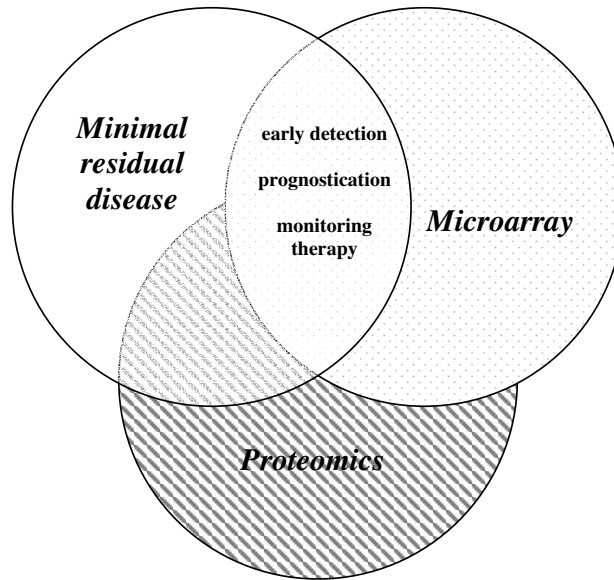


Figure 2. Integrating the three different research techniques will result in a better understanding of the molecular mechanisms of colorectal cancer and will facilitate translating hypotheses derived from basic science into improved patient care. The combination of the different research techniques may result in earlier detection, prognostication and treatment monitoring of colorectal cancer.

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