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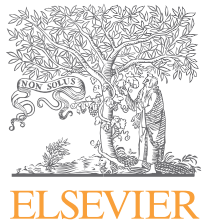

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Identification, validation and clinical implementation of cancer biomarkers: Translational strategies of the EORTC PathoBiology Group

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ABSTRACT

The increasing demand for personalized cancer therapy requires a strong, intense, and continuous collaboration between pre-clinical and clinical investigators. As a part of the EORTC Translational Research Divison, the EORTC PathoBiology Group (EORTC PBG), focuses on discovery and validation of cancer biomarkers, providing both scientific evidence as well as quality assurance. The clinically relevant target-identification and validation studies carried out in the last decades within the EORTC PBG represent a paradigm for EORTC studies in which laboratory investigations on human biologic material are used to support the development of drugs directed to defined target molecules. The experience acquired within the EORTC PBG with respect to standardization of cancer biomarker test kits and reagents, quality assessment/assurance of cancer biomarker determinations,

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development of standard operating procedures for assessment of these markers as well as instruction of methodologies and teaching of ethical issues represent a valuable contribution of the EORTC PBG to the onco-translational strategies of the EORTC.

1. Introduction

In recent years, the sharp increase in knowledge on the mechanisms involved in tumor initiation and progression coupled with our understanding of the molecular biology of cancer cells and the rapid development of high-throughput technologies have broadened the scenario of biological information related to specific cancer cell alterations/functions which can integrate clinico-pathological staging and the development of novel effective therapeutics targeting specific molecular pathways. These features are currently driving the clinical need for planning molecular-based therapies, the dawning of a new era of personalized cancer medicine where selection of type and course of treatment can be tailored to an individual's disease and genotype.[1] This innovative approach may not only increase therapeutic efficacy, but also decrease toxic side effects of cancer therapeutics and reduce costs to society and health care systems by avoiding ineffective or harmful treatments.

The most important message derived from decades of translational research (TR) in cancer relies on focusing on the clinically relevant needs, with a strong, intense, and continuous collaboration between pre-clinical investigators and physician scientists. Correct assessment of patient status and therapeutic needs may require the consistent and accurate determination of key molecular parameters and takes advantage of systematic preservation and access to biospecimens. Studies on human specimens are essential in the still ongoing process of discovering new mechanisms involved in causing cancer or in determining its progression, resistance/response to treatment, and thus clinical outcome. In particular, independent molecularly driven multicenter clinical studies form an important link for validating the clinical relevance of cancer biomarkers, thus bridging the translation of research results into clinical practice.[2]

The EORTC-PathoBiology Group (EORTC PBG), formed in 2006 from a merger between the former EORTC Pathology Group and the EORTC Receptor and Biomarker Group (RBG, operating since 1972 within the EORTC to provide knowledge on the basics and clinical exploitation of biomolecular markers/molecular signatures in tissue, cells and bodily fluids), is very active in the area of discovery and validation of cancer biomarkers.[3]

Optimization of opportunities to generate new hypotheses for TR definitely relies on a "round trip" model, with a clinical translation of pre-clinical findings coupled with pre-clinical investigations on information derived from clinical trials. This pathway towards personalized medicine is complex and warrants careful upfront planning and input from a range of expertises. The EORTC PBG supports the EORTC Translational Research Division (TRD) and Disease-Oriented Groups (DOG) with its basic science and clinical expertise to identify and validate clinically relevant cancer biomarkers/molecular signatures. The group members, with their expertise in biochemistry, cellular and molecular biology, statistics, pathology and clinical trials, are well grounded in biobanking, pre-analytical/analytical testing and assay performance as well as in statistical evaluation of the test results needed for the integration of high-quality biomarker assessment into clinical practice. In fact, the repertoire of potential cancer biomarkers is steadily increasing as is the variety of methods used for their measurements, although their standardization and quality control is often lacking as are guidelines on interpretation of test results. This fact is not always known to clinicians or to basic scientists, and even worse, assay results from inadequately validated biomarker studies are repeatedly being made available to the public in scientific publications or promoted as commercial products. Setting up quality-based guidelines and informing the medically trained cancer researcher represent a priority for the EORTC PBG.

2. Biomarker discovery and validation: recent achievements of the EORTC PBG

TR is an essential component of the EORTC PBG mission in order to assess and qualify the scientific and clinical value of biomolecular markers/molecular signatures in human biological materials (HBM), and to carry out studies to provide relevant information for patient management with high level of evidence for clinical implementation.[4] Specifically, EORTC PBG has been involved in TR activities focused mainly on solid tumors, with the most prominent being cancer of the breast, colorectum and ovary, and glioblastoma, in the context of discovery and validation of function-related biomarkers involved in oncogene or oncosuppressor gene deregulation, cell cycle and apoptosis regulation, DNA repair, extracellular matrix and neo-angiogenesis features, cell progression and dissemination to other organs in the body. Instrumental to the EORTC PBG translational model are: (a) availability of well-annotated

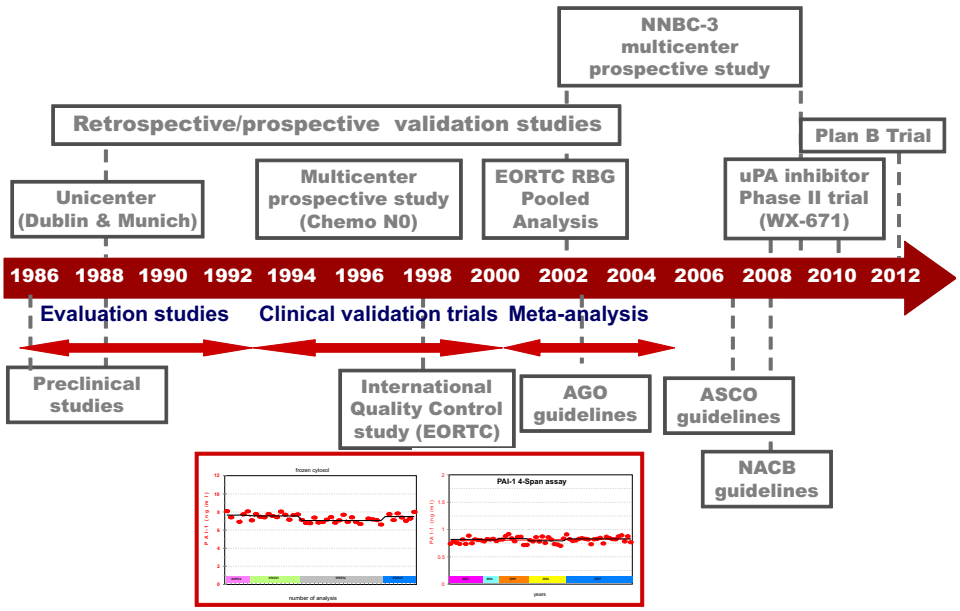

Fig. 1 – uPA/PAI-1 in primary breast cancer: an example of a successful development of a biomarker by the EORTC-PathoBiology Group (formerly EORTC-Receptor and Biomarker Group).

and high-quality biological specimens from patients entered clinical trials, through multicentric/multinational intergroup collaborations; (b) establishment of standardized, reproducible and robust assays to detect molecular cancer biomarkers/signatures even on limited tumor material; (c) quality assessment and assurance of reagents, test systems (including pre-analytical processing and post-analytical data processing), commercial kits and equipments; (d) prospective planning of the study design for the assessment of biologically and/or clinically relevant biomarkers/signatures, according to national regulatory and ethical issues.

The clinically useful PBG studies best suited to illustrate the translational strategy that leads from biomarker identification to assessment of its clinical benefit are the investigations on urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1, plasminogen activator inhibitor type-1) in breast cancer (BC).[5] Following the seminal retrospective observation on a small number of cases made by Duffy et al. in 1988,[6] that women whose BC presented at diagnosis with high levels of uPA proteolytic activity had a high risk of developing metastasis, independent but coordinated series of preclinical investigations were initiated to assess and qualify the clinical value of uPA and PAI-1 as predictors of tumor invasiveness and metastatic potential, also taking advantage of the complementarity expertise of the EORTC PBG (then still the EORTC-RBG) members (Fig. 1). Those initiatives included: (1) establishment of a standardized, quality-assured immunometric assay (ELISA) to determine uPA and PAI-1 antigen levels in tumor tissue extracts[7]; (2) pilot studies which confirmed the prognostic relevance of uPA and PAI-1 protein content in a small number of prospectively collected tumor specimens[8,9]; (3) external quality assessment within the EORTC-RBG framework of trans-European multicentric uPA/PAI-1 antigen determination in human BC tissue extracts, before converting it into a commercially available format[10,11]; (4) prognostic relevance of uPA/PAI-1 confirmed by multicentric studies carried out within the EORTC RBG in large numbers of tumor specimens analyzed retrospectively[12–14]; (5) prognostic relevance confirmed in prospectively collected tumor specimens and from an EORTC RBG pooled analysis of multicentric studies comprising more than 8,000 BC patients in order to get ready for a large clinical trial[7,15]; (6) validation of the clinical utility of the two biomarkers by a prospective high-powered randomized chemotherapy trial specifically identifying node-negative BC patients who benefit from chemotherapy, i.e., those with high levels of uPA and/or PAI-1 (Chemo-N0, level-of-evidence 1 study carried out within the EORTC PBG framework, with the participation of 14 different Institutions).[5,16]

The outcome and impact of these studies are as follows[5]:

(1) uPA and PAI-1 were included in the guidelines of the German Working Group in Gynecological Oncology, AGO (since 2002 *for prognostic assessment in node-negative breast cancer*), of the European Group on Tumor Markers[17] (since 2005), of the American Society of Clinical Oncology[18] (since 2007, "*to determine prognosis, for treatment planning and to guide use of cyclophosphamide-methotrexate-fluorouracil-based adjuvant chemotherapy*") and of the National Academy of Clinical Biochemistry Laboratory Medicine Practice (*since 2008*) for use as biomarkers in BC management[19];

(2) two additional multicentric prospective clinical trials in high-risk (uPA and/or PAI-1 high content) node-negative BC patients [20] (NNBC-3: n = 4149) or in HER2-negative BC (Plan B Trial: n = 2484, in which molecular-based risk is assessed according to Recurrence Score and uPA/PAI-1) were activated and have recently closed accrual;
(3) Phase I and II clinical trials with a small molecule inhibiting uPA (WX-UK1) have been completed in patients with BC and pancreatic cancer for which promising results in terms of prolonged time to progression and survival were reported. [21,22]

Additional examples to summarize past and current activities of the EORTC PBG for cancer biomarker discovery associated to tumor progression and metastatic potential include:

- development of "*in vivo* proof of concept" for tissue inhibitor of metalloproteinases-1 (TIMP-1), including prospective studies to evaluate the clinical significance of plasma levels for early recurrence in colorectal cancer (CRC) and as a marker for minimal residual disease in CRC and BC patients [23,24];
- investigations on molecular markers associated with invasion and metastasis (kallikrein-related peptidases, cathepsins in addition to other extracellular matrix markers), [25] with hypoxia and angiogenesis (hypoxia-inducible factor-1α, carbonic anhydrase IX), [26] and with cell survival (apoptosis and telomere maintenance mechanisms) [27] in different tumor types;
- identification of gene expression signatures that mediate BC metastasis in different sites (bone, [28] lung, [29] brain [30]);
- identification of microRNA signatures associated to metastatization in specific BC molecular subtypes. [31]
- Within this area of research, focused on biomarker discovery associated with tumor progression, recent efforts of PBG members have been dedicated to evaluate new technologies for the detection and molecular characterization of circulating tumor cells in the peripheral blood of BC patients. [32]

Once identified, biologically relevant cancer biomarkers/signatures of potential clinical interest enter the validation pathway which generally involves multicentric collaborations among PBG members. Within this context, the following validation studies have been completed or are in progress regarding:

- the Rotterdam 76-gene profile for lymph node-negative BC patients receiving local-regional treatment and/or adjuvant tamoxifen [33,34];
- genomic and proteomic projects on different cancer types to challenge gene signatures and epigenetic biomarkers with patient's prognosis and response to chemo/endocrine therapy [35–37];
- studies on the DNA-methylation profile of CpG islands within promoter regions of candidate genes in BC and brain tumors of patients subjected to different types of systemic treatments. [38–40]

In the context of the emerging scenario of personalized cancer medicine, the EORTC PBG has recently focused on pathway/target oriented strategies, with the development of experimental models for *in vitro–in vivo* studies and the establishment of functional studies for target identification and validation, also including targeting of radioresistance-related pathways for the modulation of radiation response.

3. EORTC PBG quality assessment and quality assurance programs

For proper implementation of a biomarker in clinical practice, an evidence-based consensus on its effective clinical usefulness is needed. However, clinical studies on biomarkers frequently report conflicting results. Among the factors contributing to discrepancies among different clinical studies are (1) lack of standardization of the pre-analytical procedures, including tissue sampling/collection, transport and processing in the laboratory; (2) substantial differences in methodologic approaches, test reagents and signal detection; (3) differences in statistical methods employed, including determination of cut-off points and weighting of the clinical impact of biomarkers by uni- and multivariable analyses; (4) heterogeneity of tissue architecture or molecular structure/function.

The EORTC PBG Quality Assurance (QA) center in Nijmegen has long-standing experience in this area, starting in 1979 by performing QA studies on cancer biomarkers both within and outside the EORTC. [41] Quality assessment and assurance of the reagents and test systems employed (including pre-analytical processing and commercial kits for existing markers, and accounted for collaborations with companies) [42,43] and of the equipment used to quantify the expression level of cancer biomarkers in tumor tissue extracts or in other body fluids still represent an important task of the EORTC PBG.

The EORTC PBG regularly advises clinicians and basic researchers on common methodologies for cancer biomarker assays and ensures that appropriate external quality assessment schemes are available. As most of the phase II/III cancer trials involve multi-center cooperation, special emphasis by the EORTC PBG is placed on quality and performance of the assays, on their reproducibility and on the collaboration with clinical groups of the EORTC, with partners within framework programs of the European Commission, with Health Authorities and other national and international bodies that have scientific and social interest in the supply of optimal information about quality and performance of

cancer biomarker tests and their relevance for cancer patient management.

Guiding principles are provided and discussed on how to inform physician scientists and cancer researchers about quality control systems to enable a consistent assessment of the clinical value of cancer biomarkers. Knowledge on the good laboratory practice for tumor tissue or blood sample collection and storage, tissue processing, assay methods, reference materials, and Standard Operating Procedures (SOP) is often missing. Therefore, the EORTC PBG advises on procedures, test kits and reagents to be utilized for cancer biomarker determinations. Thus, the EORTC PBG takes an active part in advising the EORTC-DOG regarding tissue/blood storage and handling, cancer biomarker assessment, SOPs,[44] biochemical/cellular methodologies and technologies as well on matters concerning good laboratory practice, quality assessment and QA. The EORTC PBG is therefore continuing to: (1) focus attention on cancer biomarker development and clinical implementation, including the development of test assays on archival specimens[39] and/or on limited amount of tumor material[45]; (2) support high-level QA programs when assessing cancer biomarkers.[46]

The current portfolio of cancer biomarkers under QA procedures by the EORTC PBG includes: (1) steroid hormone receptors, invasion factors uPA and PAI-1[11] and oncogene HER2 in BC samples; (2) assessment of circulating tumor cells in peripheral blood of cancer patients; (3) development of internal and external reference materials for cancer biomarker assays; (4) writing and communication of SOPs on specimen handling and processing.

4. EORTC PBG translational strategies: biobanking and biomarker qualification

Access to HBM, such as tumor tissue, blood, ascitic fluid, saliva, urine and cell derivatives such as RNA and DNA, in both sufficient quantity and quality, is considered a key element for cancer biomarker discovery, validation and for molecularly driven clinical trial design in order to achieve personalized treatment of cancer patients. Clinical trials offer a unique opportunity to collect HBM combined in a specialized setting that allows prospectively designed, high-quality TR that would be difficult to obtain from community- or population-based HBM collections alone. Increasingly, as the field advances towards individualized treatment of cancer patients, access to HBM is becoming a necessity for patient enrolment in a new generation of clinical studies that are designed and driven by molecular hypotheses. Hence, collection of HBM and its appropriate storage in biobanks is critical for innovative translational and clinical research, and failures/inefficiencies represent major bottlenecks hindering successful bench to bedside translation. Consequently, the EORTC has given priority to setting clear guidance and policy for addressing the challenges of pan-European HBM collection in EORTC clinical studies and to maintain public trust in biobanking for cancer research.[47] Within this context, the EORTC PBG – based on the long-term experience and expertise gained by its members who are often in charge in their institutions of large biobanks, including well-maintained frozen tumor tissues, with parallel paraffin blocks and serum/plasma collections also linked to clinical and follow-up databases – assisted EORTC with a strong and intense collaboration between its members and key persons of EORTC Translational Research Unit and HeadQuarters (HQ) in developing strategies to support the practical aspects of collection, storage, and access to HBM. The first version of the new policy to regulate biospecimen collections in EORTC studies was released in January 2011. The principles of the new EORTC policy promote best practice in biobanking and are in accordance with current international legal and ethical standards for storage and future use of HBM. Key topics covered include: (1) coordination of the chain of custody of HBM collected from patients enrolled in EORTC clinical studies; (2) ethical principles of coordinating trans-border collections of HBM; (3) confidentiality and data protection; (4) management of and access to HBM; and (5) publication of resulting research. To simplify TR integration and HBM collection in clinical studies, the EORTC PBG supported the EORTC in developing a checklist containing the key elements of HBM collection set-up and combining these into a simple tool for practical use. Through identification and managing of key risk areas, the success of HBM collection can be maximized whilst achieving efficient clinical trial development. The EORTC PBG is also participating in collaborations with other Societies and organizations, notably the European Society of Pathology, to stimulate the involvement of pathologists in EORTC clinical studies and to assist with the biobanking and quality assurance strategies.

5. EORTC PGB transfer-of-knowledge programs

Transfer-of-knowledge from EORTC PBG centers of excellence to other EORTC groups represents an important task, that can be achieved through: (1) transfer of knowledge about cancer biomarker utility in the clinical setting[48]; (2) teaching various aspects of basic and clinical research regarding biology and clinical impact of cancer biomarkers and (3) transfer results on the biological importance of cancer biomarkers obtained in the research setting to the clinic (Fig. 2). The complementary expertise of the various EORTC PBG members makes them particularly suited to teach

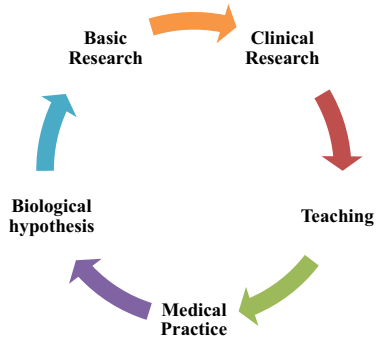

Fig. 2 – Interrelation of basic and clinical cancer research, teaching and medical practice.

different aspects associated with the clinical utility of cancer biomarkers, the different approaches for their characterization, quantification and validation, and the quality assurance/assessment aspects. All these tasks represent the main topics discussed at the annual EORTC PBG meeting, and EORTC PBG members are instructed at workshops, teaching classes and symposia about methodological approaches and protocols regarding the clinical implementation of important cancer biomarkers. Furthermore, information is provided regarding the design and execution of animal studies and (pre)clinical trials, including ethical, legal, and societal aspects of performing cancer research at the basic and/or clinical level.

EORTC PBG members are organizing and participating in the alternate annual EORTC–NCI–ASCO (ENASCO) meetings in Brussels and the USA, and in the annual tutorial for young clinical investigators held in conjunction with the ENASCO meeting. Moreover, continuous knowledge exchange across the Atlantic is guaranteed by a NCI liason officer within the EORTC PBG. [46]

6. Future strategies

Within the frame of the implementation program of TR promoted by EORTC, EORTC PBG will contribute to several aspects, including improvement of interactions with DOGs and Network of Core Institutions (NOCI), thus providing constant support to EORTC HQ for issues concerning biobanking activity and review of TR studies/protocols in order to stimulate ideas for research and advice on HBM collections. At the same time, EORTC PBG will assist EORTC in methodology validation, by extending Quality Control programs to provide support to EORTC TR trials, e.g., on circulating tumor cells, RNA-based biomarkers and mutation analysis, and for pathway/target oriented strategies, through the availability of experimental models for *in vitro–in vivo* studies, for target identification and validation, and for functional studies. In this complex scenario derived from an increasing demand for individualized cancer therapy, the experience acquired within the EORTC PBG during several decades of collaboration among its members will be of value for the process of incorporation of cancer biomarkers into novel early clinical trial designs. Eventually, this will allow optimal evaluation of development of refined monitoring techniques and tools, biological assays and test reagents suitable for *in vitro* and *in vivo* assessment of cancer biomarkers, and will assure a high-quality laboratory infrastructure and expertise with the capability to timely provide biological readouts on HBM. To comply with the requirements of the novel molecular medicine, an intensification of collaborations (Fig. 3) will be instrumental for a better tailoring of EORTC PBG activities according to EORTC needs. In keeping with this approach, in addition to NCI and ASCO liaisons and to the participation of PBG members on behalf of EORTC or of their own Institutions in several European Union-funded framework projects (including BBMRI and EurocanPlatform), the initiation of new collaborations such as with the European Society of Pathology (for a full integration of pathological issues and investigations into contemporary clinical trials) and with the Sanger Institute (for comprehensive profiling of tumor somatic mutations, linking cancer biomarker results with molecularly stratified clinical trials and developing a molecular electronic record system to allow patient access to forefront tailored treatments and to foster the collaboration with pharma industries) will

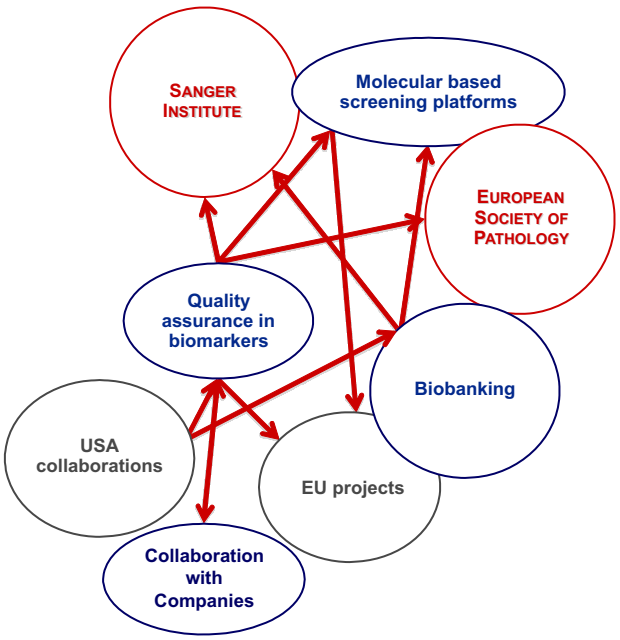

Fig. 3 – A network diagram illustrating the interrelationships among the EORTC PBG key areas of interest (blue circles), as well as ongoing (grey circles) and in-progress (red circles) collaborations.

strenghten the participation of EORTC PBG in the EORTC strategy and foster the collaboration of EORTC with the pharma industry.

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8. Conflict of interest statement

Maria Grazia Daidone, John A. Foekens, Nadia Harbeck, John Martens, Nils Brunner, Jacqueline A. Hall, Roberto Salgado, Juergen Dittmer, M. Joe Duffy, Fred C.G.J. Sweep, Manfred Schmitt and Anneke Geurts-Moespot have no conflicts of interest. Christoph Thomssen received honoraria and research funds from American Diagnostica.

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