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Gene expression analysis in biomarker research and early drug development using function tested reverse transcription quantitative real-time PCR assays

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ABSTRACT

The identification of new biomarkers is essential in the implementation of personalized health care strategies that offer new therapeutic approaches with optimized and individualized treatment. In support of hypothesis generation and testing in the course of our biomarker research an online portal and respective function-tested reverse transcription quantitative real-time PCR assays (RT–qPCR) facilitated the selection of relevant biomarker genes. We have established workflows applicable for convenient high throughput gene expression analysis in biomarker research with cell lines *(in vitro* studies*)* and xenograft mouse models *(in vivo* studies*)* as well as formalin-fixed paraffin-embedded tissue (FFPET) sections from various human research and clinical tumor samples. Out of 92 putative biomarker candidate genes selected *in silico*, 35 were shown to exhibit differential expression in various tumor cell lines. These were further analysed by *in vivo* xenograft mouse models, which identified 13 candidate genes including potential response prediction biomarkers and a potential pharmacodynamic biomarker. Six of these candidate genes were selected for further evaluation in FFPET samples, where optimized RNA isolation, reverse transcription and qPCR assays provided reliable determination of relative expression levels as precondition for differential gene expression analysis of FFPET samples derived from projected clinical studies. Thus, we successfully applied function tested RT–qPCR assays in our biomarker research for hypothesis generation with *in vitro* and *in vivo* models as well as for hypothesis testing with human FFPET samples. Hence, appropriate function-tested RT–qPCR assays are available in biomarker research accompanying the different stages of drug development, starting from target identification up to early clinical development. The workflow presented here supports the identification and validation of new biomarkers and may lead to advances in efforts to achieve the goal of personalized health care.

1. Introduction biomarker research

Personalized health care (PHC) uses genetic information to understand requirements for health maintenance, disease prevention, and therapy. The aim of PHC is to select the right medicine at the right dose for the right patient at the right time [1–3]. The identification and characterization of molecular biomarkers, used as indicators for a certain biological state, plays an important role in PHC [4,5]. Biomarkers may serve for disease prognosis, the prediction of therapeutic response and monitoring duration and quality of response. Thus biomarkers have the potential to support clinical decisions, from diagnosis to treatment planning, to improve tailored treatment strategies to avoid over- or under treatment and adverse side effects and finally to enhance prognosis and cost effectiveness. For several years, the integration of biomarkers in drug development and their use as companion diagnostic in clinical practice is also strongly encouraged by regulatory authorities [6,7]. Therefore, biomarkers have become a central element in a drug's lifecycle, from target identification throughout pre-clinical and clinical development up to the application of the launched drug (Fig. 1).

Abbreviations: ALAS1, Aminolevulinate, delta- synthase-1; Cq, quantification cycle; FFPET, formalin-fixed paraffin-embedded tissue; HER2, human epidermal growth factor receptor 2; HPRT1, hypoxanthine phosphoribosyltransferase-1; PHC, personalized health care; qPCR, quantitative real-time PCR; RT–qPCR, reverse transcription qPCR; TGI, tumor growth inhibition.

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For example in breast cancer which displays extensive molecular heterogeneity the human epidermal growth factor receptor 2 (HER2/neu) represents a well established predictive molecular biomarker to differentiate HER2-overexpressing tumor subtypes in order to allow HER2 targeted therapy [4,5,8,9]. Today advancements in high throughput technologies significantly facilitate biomarker identification and constitute the basis to take full advantage of the potential biomarkers offer for PHC. Currently efforts are undertaken to standardize biomarker discovery, development and validation in order to rise the percentage of biomarkers passing the barriers for clinical application [1,10].

As fresh-frozen clinical tissue samples are largely limited as source for biomarker research the application of formalin-fixed paraffin embedded tissue (FFPET) specimens becomes important for gene expression analysis from pathology samples [11]. Formalin-fixation and paraffin embedding (FFPE) is the clinical standard for tissue fixation and processing for the purpose of diagnostic histology and long-time storage. For instance, in the clinical diagnosis of cancer patients FFPET samples have been routinely obtained for decades. Therefore, the existence of huge archives of FFPET specimens worldwide constitutes a valuable source of retrospective long term biological material for follow up biomarker discovery and validation and may provide material to sustained progress in the development of PHC.

In the present study we describe the use of function-tested RT–qPCR assays in order to discover appropriate biomarkers during pre-clinical and early clinical studies. In biomarker research, the investigation of mRNA expression profiles in the context of complex biological pathways is of great interest and represents a common initial step in the detection and identification of suitable biomarkers. The RT–qPCR technology is known to be highly specific and sensitive and in these aspects superior to the microarray technology [12–14] but the number of evaluated genes at one time is rather limited. Therefore, the goal of this study was also to establish a workflow offering high throughput and application convenience for efficient expression profiling.

In order to facilitate the selection of relevant biomarker genes as well as gene expression profiling we used a specialized online portal (RealTime ready Configurator, Roche Diagnostics) and the respective function tested RT-qPCR assays (function tested RealTime ready RT-qPCR assays). The gene panel selected using this tool (provided as a RealTime ready custom panel) were analysed by RT-qPCR on the LightCycler® 480 instrument for which the respective pre-plated assays were available. This study describes established workflows applicable for analysis of differential gene expression in biomarker research with cell lines (*in vitro*) and xenograft mouse models (*in vivo*) as well as with FFPET sections from various human research samples.

2. Overview of biomarker development workflow

The methodology applied to identify suitable biomarkers for the development of a therapeutic antibody compound is summarized in Fig. 2. For the target-specific therapeutic antibody the biology and the down stream signal transduction pathways were analysed using literature search and microarray data (mRNA expression data, unpublished in-house data). These *in silico* analyses revealed the involvement of the NF-κB pathway. Based on these results, we assembled a special panel of 92 NF-κB related genes of putative interest using the web portal (https://configurator.realtime-ready.roche.com) with the "keyword" search function.

To generate a first hypothesis, the NF-κB panel was used for *in vitro* gene expression analysis in nine different human tumor cell lines using function-tested RT-qPCR assays. To confirm potential biomarkers, based on the cell line results, we applied a reduced NF-κB panel (35 parameters) to *in vivo* mouse xenograft models. Based on the results obtained from the cell line and xenograft measurements, 13 potential candidate genes were identified. To test the hypothesis, six of these parameters were selected to analyze human FFPET samples. For these six parameters and selected reference genes, suitable for expression analysis in the tumor entities of interest, respective RT-qPCR assays were specifically developed and optimized to function with RNA isolated from FFPET. The aim of the preclinical and early clinical data generation was to select the most appropriate biomarkers for prospective hypothesis testing and ultimately for potential companion diagnostics (Dx) development.

3. Description of methods

3.1. Biomarker hypothesis generation in vitro: Workflow description for cell line studies

The workflow for *in vitro* cell culture studies with the selected NF-κB panel is described in Fig. 3 comprising the steps cell culture and sample preparation, RNA isolation, cDNA synthesis and qPCR. This workflow is designed for high throughput analysis required at the initial biomarker development step of hypothesis generation.

3.1.1. Cell culture

Commercially available tumor cell lines selected based on target receptor expression (1: human renal cell carcinoma; 2: human renal cell carcinoma; 3: human osteosarcoma; 4: human pancreatic carcinoma; 5: human breast cancer; 6: human pancreatic carcinoma; 7: human lung carcinoma; 8: human lung carcinoma; 9: human prostate carcinoma) were cultured under standard conditions. The cells were treated with recombinant target for the therapeutic antibody or the therapeutic antibody, respectively. The tumor cell lines were harvested at different time points (0, 6, 24 h) and immediately resuspended in RLT buffer (Qiagen) and stored at – 80 °C up to several months.

3.1.2. RNA Isolation from cells

Automated isolation of total cellular RNA was performed from the cell pellets using the MagNA Pure LC Instrument with the

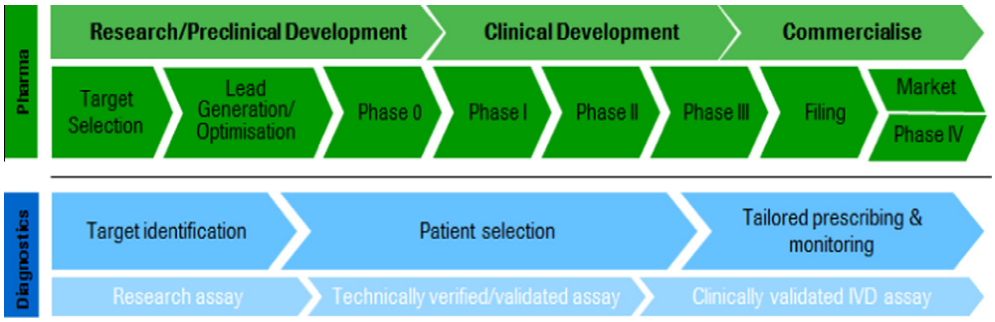

Fig. 1. Biomarkers are relevant for the entire development cycle of a therapeutic compound from target identification to drug application.