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Combined multi-gene analysis at the RNA and protein levels in single FFPE tissue sections

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ABSTRACT

Novel approaches of individualized medicine require rapid analyses of comprehensive multi-gene expression patterns both at the RNA and protein levels. Optimally these analyses are achieved with minimal amounts of tissues, which are derived from routine procedures of clinical diagnostics. We demonstrate the parallel analyses of gene expression of six different genes at the RNA and protein levels in two consecutive sections of routinely processed FFPE tissues. This was achieved by combination of multi-epitope-ligand cartography (MELC) and fully automatically magnetic bead-based RNA extraction and subsequent qRT-PCR analysis. Our work provides proof-of-principle that comprehensive analyses of multi-gene expression patterns can be achieved by the combination of these two high content technologies. This may provide new perspectives for the determination of pathogenic gene expression in the framework of individualized medicine.

Introduction

The number of molecular imaging targets continues to increase and there is a constant need to reduce sample handling, simplify workflow and in cancer research additionally a need to characterize multiple intracellular targets (Levenson et al., 2008). Existing technologies utilize dye labeled antibodies to characterize cellular components and to quantify targets of interest; however an upper limit of four targets has been reported (Camp et al., 2002). The multi-epitope-ligand cartography (MELC) technology is a fluorescence microscopy based method for the measurement of multiple proteins. It is a digitalized immunofluorescence technique which uses the sequential staining of one section with up to 100 different antibodies (Schubert et al., 2006). There is an automated cycle of staining the sample with fluorescence labeled antibodies, imaging and finally photo bleaching of the label. In each cycle an antibody specific for a different protein can be applied so that the result is a set of images of the distributions of many different proteins for the same field. As yet, the MELC technique was exclusively applied to frozen tissues.

In order to allow multi-gene analyses at the RNA level in the framework of clinical routine processes recently automated procedures have been introduced allowing the extraction of RNA from routine formalin-fixed paraffin-embedded (FFPE) tissue material, which can be used for subsequent quantitative reverse-transcription PCR (qRT-PCR) approaches (Bohmann et al., 2009). With this approach sufficient amounts of RNA can be extracted from a single section for up to 500 different gene expression analyses (Müller et al., 2011; Petry et al., 2008).

Molecular diagnostics of individualized medicine requires methods allowing the comprehensive parallel analyses of many different proteins at the single cell level and quantitative determination of the respective gene expression at the RNA level. In order to address this demand, the goal of this study was to combine a high content immunohistochemical approach (MELC) with automated RNA extractions and quantitative PCR approach on single tissue sections. We applied this strategy for the first time on routinely processed FFPE tissues of melanoma (MM) and colorectal carcinoma (CRC).

Material and methods

Tissue preparation

Tumor tissue from CRC or MM was routinely fixed in neutrally buffered formalin for 24 h and was then dehydrated and embedded in paraffin. In order to provide standardized conditions for the staining procedure, CRC and MM tissues were embedded next to each other in one block. For MELC analysis one 5-µm section was cut and applied on a silane-coated slide. To deparaffinize the samples, the sections were treated with xylene, followed by ethanol washes. Further the specimen was incubated with 5% NGS (Dako, Hamburg, Germany)

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in PBS (PAA, Pasching, Austria) for 30 min in order to block unspecific binding sites. For RNA extraction the CRC and MM tissue of two consecutive 5-μm sections (the sections were also consecutive to the MELC section) were separated and were placed in micro-centrifuge tubes.

RNA isolation from FFPE tissue samples

Total RNA was isolated from two 5-μm whole FFPE tissue sections of CRC or MM samples with a fully automated method based on silica-coated iron oxide beads (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) (Bohmann et al., 2009; Wolf et al., 2012). In the last step an automatic DNase I digestion was also performed on the system.

Primer selection

qRT-PCR primers and 5′-FAM–3′-TAMRA-labeled hydrolysis probes were selected using Primer Express® Software Version 3 (Applied Biosystems, Life Technologies, Karlsruhe) according to the manufacturer's instructions. The absence of single nucleotide polymorphisms and the specificity of the selected primers were verified by BLAST. All primers and probes were obtained from Eurogentec S.A. (Seraing, Belgium). Primer and probe sequences are shown in Supplementary Table 1. Primers and hydrolysis probes were diluted to 100 μM for stock solution with nuclease-free water (Qiagen, Hilden, Germany). For qRT-PCR 0.5 μM of each primer and 0.25 μM of each probe were used.

Gene expression analysis with reverse-transcription quantitative PCR

All quantitative one-step reverse-transcription PCRs (qRT-PCR) were performed in triplicate using the SuperScript® III Platinum® One-Step qRT-PCR kit with ROX (#11732088; Life Technologies) according to the manufacturer's instructions, except for a longer reverse-transcription period of 30 min at 50 °C. For evaluation the mean of the triplicates was used. qRT-PCRs were performed on a Stratagene/Agilent MX3005P QPCR system (Agilent, Waldbronn, Germany) that was part of the Siemens VERSANT® kPCR Molecular System (software version 1.1). Forty cycles of nucleic acid amplification were applied and the baseline-corrected normalized fluorescence threshold for all primer/probe sets was fixed to 0.02. In a first step, the absence of residual DNA amounts in the undiluted RNA extractions was reviewed by a progestogen-associated endometrial protein (*PAEP*) gene-specific quantitative PCR without the preceding reverse-transcription step and with the reagents from the SuperScript® III Platinum® One-Step qRT-PCR kit with ROX and Platinum *Taq* DNA polymerase (Life Technologies). Human genomic DNA from blood was used as a positive control (Roche, Mannheim, Germany). No residual DNA in the RNA extractions was detectable. For normalization extracted RNAs were measured for the expression of the housekeeping gene ribosomal protein L37a (*RPL37a*). Subsequently RNAs were diluted with nuclease-free water to a Ct value for RPL37a of Ct23 and were stored in small aliquots at −20 °C (stocks were frozen at −80 °C). For PCR 0.5 μl of diluted patient RNA was used in a final volume of 10 μl per well.

Internal standards for quality controls

In each PCR plate (96-well plates, Agilent, #401334) a non template control (NTC) and a positive control PCR were performed using the same mastermix as employed for the patient measurements. Nuclease-free water was used as NTC and a pool of RNAs from 20 CRC patients of each UICC-stage was used as positive control. These controls were performed to compare different PCR plates measured with the same primer/probe set. Major deviations in the positive control values (delta Ct > 1 from the mean value) led to repetition of the whole PCR plate. Additionally two separate controls were included in each PCR plate. Another NTC negative and a QRef (Stratagene® QPCR Human Reference Total RNA, Agilent) positive control – not prepared out of the same mastermix used above – were measured for RPL37a to prove consistency over all PCR plates.

MELC library

A MELC library of 19 fluorophore-labeled antibodies including propidium iodide as a nucleic acid dye was used (Supplementary Table 2). The tags for the binding sites according to source, clone, working dilution, and fluorophore label are summarized in Supplementary Table 2. The appropriate working dilutions, incubation time (15 min), and positions within the MELC run had been determined in pre experiments based on MELC calibration runs.

MELC data generation

The MELC technology has been described previously (Bonnekoh et al., 2007; Eyerich et al., 2010). Dewaxed paraffin sections underwent antigen retrieval using a conventional steam boiler for 40 min with a Tris/EDTA buffer, pH 9 (Dako, Germany). The coverslip with the sample was positioned onto a motor-controlled XY stage of an inverted fluorescent microscope (Leica DM IRE2, Leica Microsystems, Wetzlar, Germany; ×20 air lens; numerical aperture, 0.7). The repetitive cyclic process of this method includes the following steps: (a) fluorescence tagging, (b) washing, (c) imaging and (d) photo bleaching. By a pipetting robot unit the fluorescence-labeled tag was applied to the sample and after incubation for 15 min rinsed with wash solution. Phase contrast and fluorescence-signal images were taken by a cooled CCD camera (Apogee KX4, Apogee Instruments, Roseville, CA; 2048 × 2048 pixels; 2× binning results in images of 1024 × 1024 pixels; final pixel size was 900 × 900 nm). Subsequent photo bleaching at the excitation wavelengths was connected downstream to delete the fluorescence signal of the applied tag. After completing this cycle, the next tag was added to the tissue sample. Data acquisition was achieved using a fully automated imaging software developed by the former company MelTec GmbH (Magdeburg, Germany). Four visual fields were recorded simultaneously in each MELC run, two of the colorectal carcinoma and two of the melanoma sample, respectively.

MELC data analysis

Using the corresponding phase-contrast images, we aligned fluorescence images produced by each tag pixel wise and corrected them for illumination faults using flat-field correction. The alignment reached a resolution of ±1 pixel. Post bleaching images were subtracted from the following fluorescence tag images. Superimposed images composed a *n* epitope expression in relation to each pixel (900 × 900 nm^2 area) of a visual field (1024 × 1024 pixels).

Results

Multi-epitope-ligand cartography (MELC) analysis was established for the use on FFPE tissue sections, using 19 different antibodies. Usually antibodies for FFPE tissue need different specific demasking protocols while for the automated technology only one protocol can be used for one section. Here we established an optimized demasking protocol which was valid for all of the antibodies used. Antibodies for pathogenetically relevant target proteins were chosen, including antibodies indicating signal transduction (Rac1, β-catenin, Notch1, Notch3, p63, Bcl2, BRaf), cell type determination (CD34, CD107a, CD45) and cell proliferation (Ki67). Using single sections from malignant melanoma (MM) and colorectal carcinoma (CRC) we could successfully establish tissue staining with these 19 antibodies (Fig. 1, MM is shown). Exemplarily a multicolor overlay of the staining with six selected markers is shown (Fig. 1, lower panel).

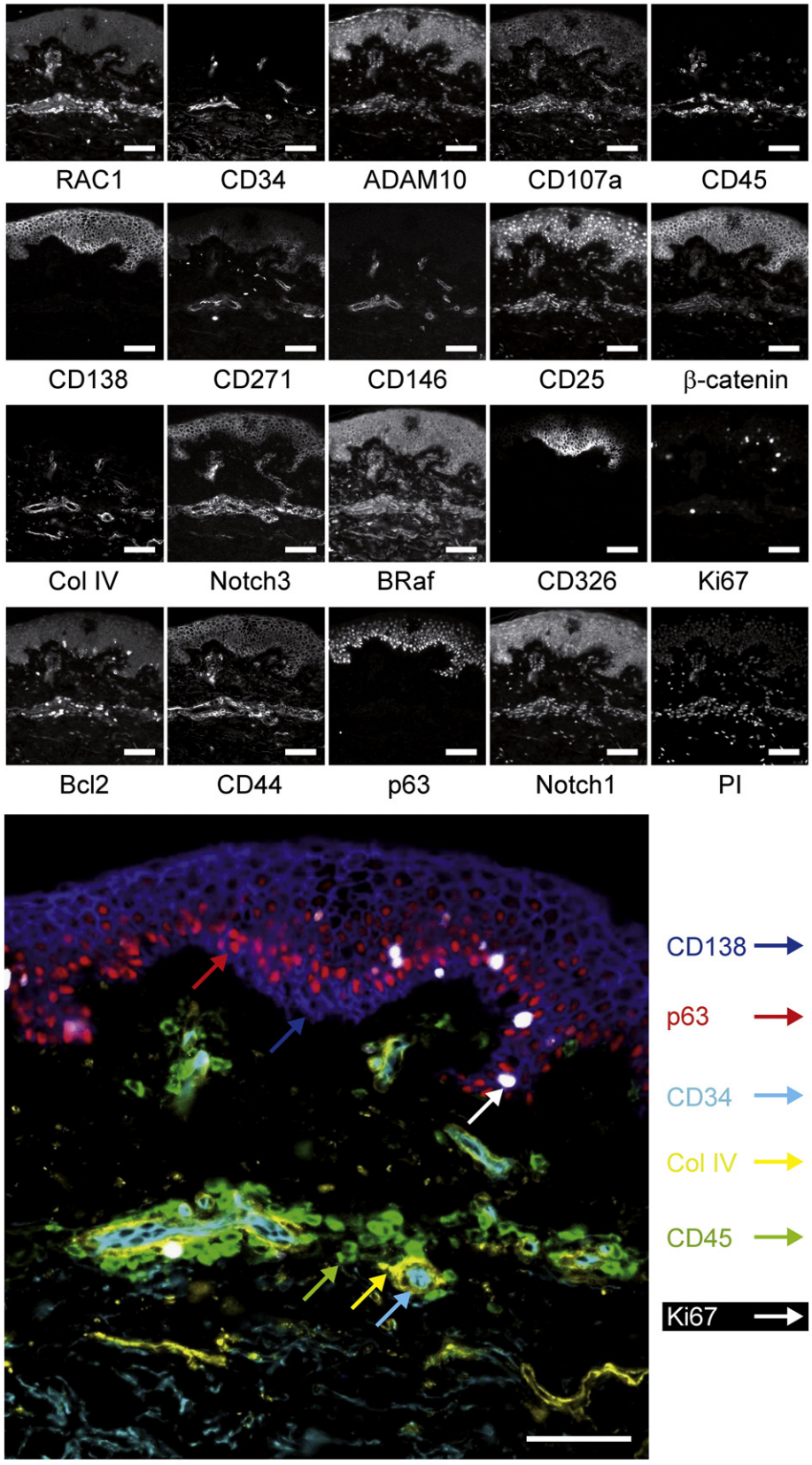

Fig. 1. Application of multi-epitope-ligand cartography with 19 different markers in FFPE tissue sections. A single section of malignant melanoma was subjected to the MELC procedure using optimized conditions for the 19 different indicated antibodies. The results for the respective stainings are shown. Cell nuclei were contrasted by propidium iodide (PI) staining (upper panels). Overlay of six selected markers in a combination picture is shown (lower panel). Different stainings are highlighted by different colors (lower right panel). Respective staining signals in the composite picture are indicated by color coded arrows. Bars = 100 μm.

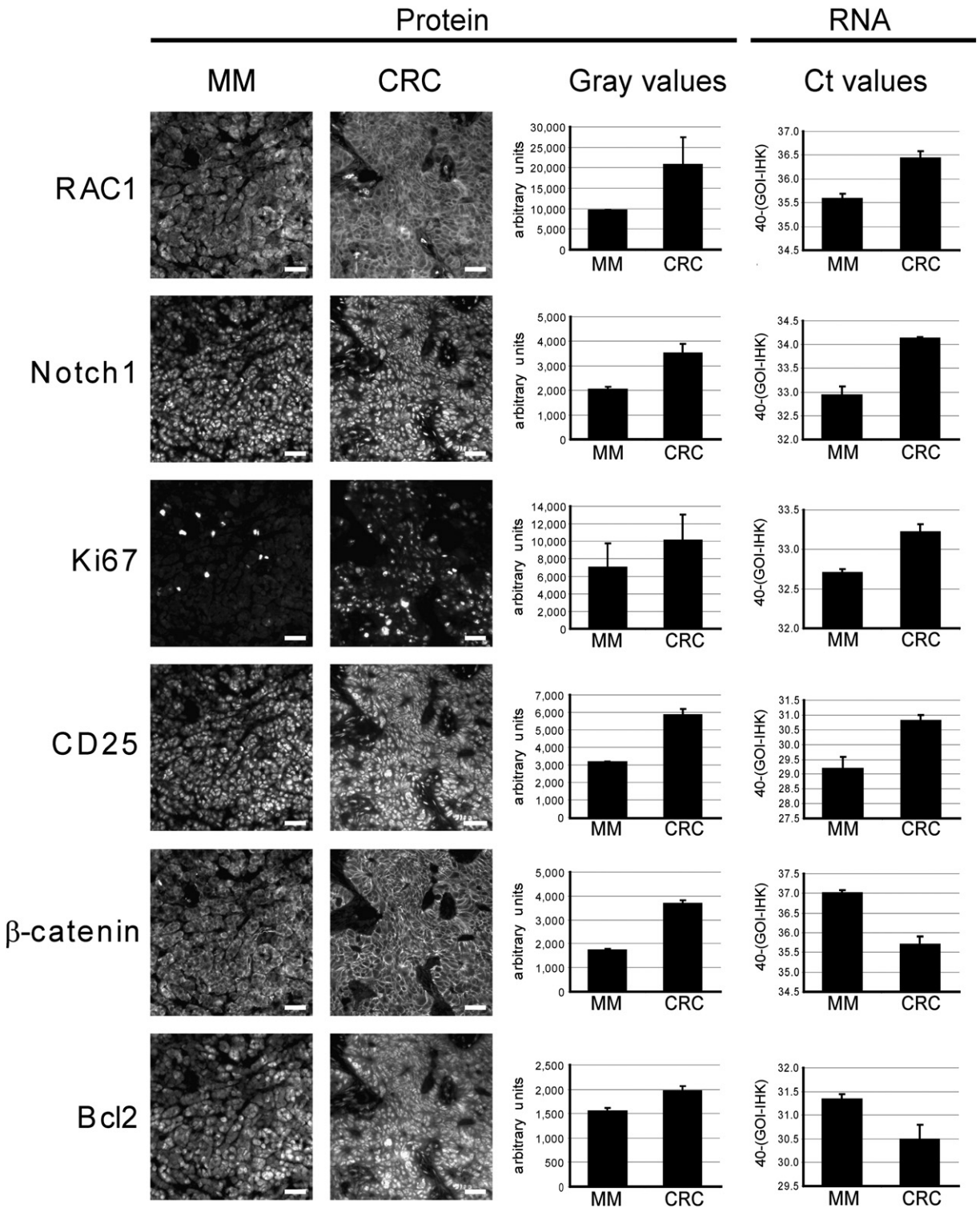

Fig. 2. Quantitative comparison of MELC and qRT-PCR from single tissue sections. Six selected marker genes were subjected to MELC, using single tissue sections from malignant melanoma (MM) and colorectal carcinoma (CRC) (left panels). Signal intensities were quantified and are shown in the bar diagrams in relative units (right panels). From a respective consecutive section of the MM and CRC tissues RNA was extracted with an automated procedure and subjected to qRT-PCR. The results obtained were normalized according to a housekeeping gene (RPL37A) and are given as relative values [40 – Ct(Gene of Interest; GOI) – Ct(Internal Housekeeper; IHK)]. Bars = 50 μm.

In a next step, consecutive sections of those subjected to the MELC procedure were applied to an automated RNA extraction procedure. Subsequently probe-based quantitative reverse-transcription PCR (qRT-PCR) was performed for the detection of the expression of six selected genes (Fig. 2, RNA). qRT-PCR results were normalized to the level of a stable housekeeping gene (RPL37A). Subsequently, signal intensities for the expressions of the different genes obtained in the qRT-PCR and in the MELC procedure in the MM and the CRC tissues were determined and compared at the quantitative levels (Fig. 2, right panels). This approach demonstrated that most of the genes are concordantly increased in CRC as compared to MM at the RNA level and at the protein level (Rac1, Notch1, Ki67, CD25). For two of the

markers (β-catenin, Bcl2) discordant results were obtained at the RNA and the protein level. In these cases immunohistochemical signals were increased in CRC tissues as compared to MM. In contrast, at the RNA level an increased level of the respective genes was observed in MM as compared to CRC (Fig. 2, right panels).

Discussion

Here we describe a proof-of-principle approach for the simultaneous analysis of the expression of many different genes both at the RNA and the protein level in only two consecutive sections of routinely processed FFPE material. The use of routine FFPE material is of great benefit, because this tissue preservation method is the routine process in clinical diagnostics. Accordingly, technologies based on this material can be easily implemented into clinical routine. Moreover, the clinical parameters are available, the fixation and the embedding procedure are standardized, tissue morphology is well preserved and tissues can be stored for many years.

We adapted the previously described MELC procedure for the use of FFPE material and combined it with an automated RNA extraction procedure from FFPE tissue sections and subsequent probe-based qRT-PCR. We show that at least 19 different pathologically relevant markers can be detected in a single FFPE section using the modified MELC procedure. With the automated RNA extraction procedure RNA can be extracted from a single CRC section (surface: 1 cm^2, thickness: 10 μm) which is on average sufficient for more than 500 qRT-PCR analyses (Müller et al., 2011; Petry et al., 2008). Combination of both techniques obviously opens new perspectives for comprehensive gene expression analyses at the protein level in single cells in combination with quantitative RNA detection. Only minute amounts of tissues are required for these comprehensive high content analyses which is a major advantage. We consider it important to make the scientific community aware of the unique potential of this approach.

Specific methodological considerations are that, as described for any other antibody-based method, also MELC requires a target protein expression above a certain detection limit. Moreover, efficiently secreted/soluble proteins may not be associated with the producing cell or may not be detected at all due to dilution effects by spreading in the microenvironment of the cell. The latter may be compensated by the analysis of the RNA in the consecutive tissue section. To determine whether tissue specific factors may affect the combined application of both techniques two different tumor types, CRC and MM, were included in this study and processed simultaneously. No tissue specific effects were observed. With the simultaneous implementation we could eliminate critical parameters like differences in antibody concentration, light intensity, etc.

The reliability of the results obtained by the MELC procedure is significantly improved as compared to classical single antigen directed immunohistochemical staining approaches as all proteins analyzed within the same tissue section are serving as additional internal controls for every other antigen examined. The use of one tissue section has however the disadvantage that antibodies which require non compatible antigen retrieval protocols cannot be combined on one section. In this framework compatible groups of antibodies may be established and processed onto two consecutive sections using two different methods of retrieval. For qRT-PCR analysis it should be considered, that RNA isolated from FFPE tissue is fragmented. Therefore the primers and probes should be designed accordingly that the PCR product does not exceed 150 bp.

In order to demonstrate that both methodologies can be combined at the quantitative level the expression of six representative genes was compared quantitatively in specimen of two major tumor diseases, CRC and MM. As expected, in the majority of cases (67%) quantitative differences between the two diseases at the protein level were clearly in agreement with differential expressions of the respective genes at the RNA level. In two cases (33%; β-catenin and Bcl2) protein signals were higher in CRC as compared to MM, whereas RNA signals were higher in MM as compared to CRC. This may indicate that the latter genes in the two diseases might be subjected to differential post-translational regulation. These results are in agreement with Ghazalpour et al. (2011) who compared the proteome and transcriptome variation in mice and reported that only 50% of the tested genes correlated significantly. Moreover it has been shown that it is insufficient to predict cellular protein levels from quantitative mRNA data, because the protein level may vary by more than 20-fold from the mRNA level (Gygi et al., 1999). Accordingly comprehensive analyses both at the RNA and protein levels are required (Ghazalpour et al., 2011).

The described combination of the two high content analysis technologies, using samples which are obtained during routine procedures in pathology, provides a significant advantage in the determination of gene expression and gene regulation in the framework of individualized medicine.

Conclusions

Here, we provide for the first time a new technology which allows high content screenings at the protein level in single cells in combination with quantitative determination of gene expression at the RNA level in single tissue sections which were obtained during routine processing in pathology. This approach will significantly contribute to improve the molecular diagnostics in the course of individualized medicine and also research activities such as detection of biomarkers in tissues.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2013.03.008.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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