Abstract #2062

Pre-analytical conditions strongly influence molecular pattern in clinical biospecimen

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Introduction

The development of personalized medicine in oncology is fostered by high-throughput analysis of molecular biomarkers in human cancer biospecimens. Tissue quality strongly depends on the pre-analytical conditions in which it was acquired. Therefore, warm and cold ischemia times that tissues are exposed to during collection are of critical importance. Insufficient quality of such tissues may lead to spurious results and data misinterpretation resulting in biased stratification of patients. The present study was conducted to gain a better understanding of the effects of warm and cold ischemia on the molecular composition of tissue specimens and especially on critical clinical biomarkers whose expression and activity level inform targeted therapy evaluation in cancer. NanoPro™1000 technology enables the differential quantification of protein isoforms and their phosphorylation status in a single measurement using very small amounts of protein. Protein can be detected at picogram levels, allowing analysis of post-translational modification in samples where limited protein is available (e.g., samples from small-needle biopsies) and making this a valid technology for the clinical setting. NanoPro™1000 has the potential to characterize protein phosphorylation of multiple sites using just one pan-antibody.

Methods

Patient recruitment:
Fifty patients with colorectal cancer (CRC) and 43 with hepatic metastasis of CRC who were scheduled for tumor resection surgery gave informed consent to be enrolled in the study. Only patients with a tumor larger than 3 cm in diameter were enrolled. Neoadjuvant treated patients were excluded. The study received approval by the competent ethics review committee of the medical association Hamburg.

RNA Extraction:
RNA was prepared in duplicates from every tissue block. RNA was isolated by two steps using phenol chloroform extraction followed by the RNeasy MinElute Cleanup Kit from Qiagen according to manual instruction.

Gene expression:
RNA samples were analyzed in biological replicates using Affymetrix chips (whole genome; Affymetrix-Power-Tools). Statistical analysis of change in gene expression was performed by cluster analysis and direct group comparison.

Immunohistochemistry:
Tissues were formalin fixed, paraffin embedded and cut into five µm thick slices. IHC stainings were conducted using the Benchmark® Ultra (Roche Diagnostics Deutschland GmbH).

NanoPro™1000 Technology:
Analysis of signaling proteins was conducted using the NanoPro1000 technology platform. This technology enables the identification of multiple isoforms according to their isoelectric point. Therefore, protein lysates were separated on a nested Premix G2 5-8 gradient against the pI standard ladder 3 and immobilized for 70 seconds.

Meso Scale Discovery:
Quantification of proteins was conducted using 96-well format technology platform from Meso Scale Discovery (MSD®, Gaithersburg, MD, USA). MSD® 96-well MULTI-SPOT® assays were performed for key proteins from the MAPK and PI3K signalling pathways.

Workflow

Study design – Sample collection

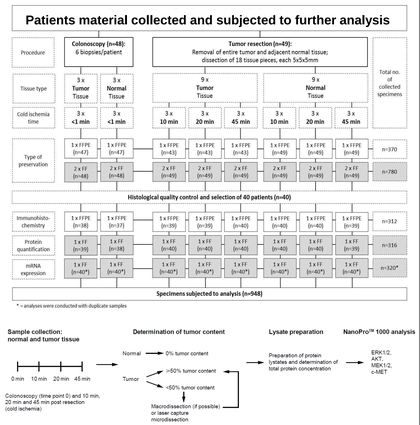

Figure 1: Flow chart showing the essential steps of tissue collection preparation and subsequent analysis. This scheme of collection and processing was applied to the colon as well as liver tissue samples.. Samples for NanoPro™1000 analysis were adjusted in tumor content.

NanoPro™1000 - Principle of technology

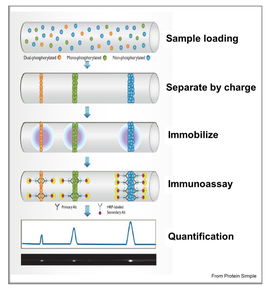

Figure 2: The NanoPro 1000 Technology enables the identification of multiple isoform phosphorylations by separating low amounts of proteins according to their isoelectric point. Therefore, by integrating this technology into our drug testing platform we are able obtain more detailed information about activation and inactivation of signaling proteins. (™ ProteinSimple)

Results

Changes in gene expression due to ischemia (Affymetrix)

		Normal colon tissue				Colorectal tumor tissue			
		Pre vs 10'		Pre vs 45'		Pre vs 10'		Pre v. 45'	
Gene	Protein and function	p-value	Log-fold change	p-value	Log-fold change	p-value	Log-fold change	p-value	Log-fold change
CYR61	Cysteine-rich angiogenic inducer 61; extracellular matrix-associated signaling protein that plays important roles in tissue repair	1.57E-18	**2.49**	2.84E-22	**2.55**	0.00000133	**1.41**	3.96E-08	**1.59**
RGS1	Regulator of G-protein signaling 1; attenuates signaling activity of G-proteins	4.21E-21	**2.34**	5.05E-25	**2.39**	1.81E-09	**1.57**	7.62E-12	**1.91**
EGR1	Early growth response 1; transcription factor	1.65E-22	**2.33**	8.96E-25	**2.36**	3.06E-09	**1.42**	5.2E-12	**1.76**
SGK1	Serum/glucocorticoid regulated kinase 1; activates potassium, sodium and chloride channels	1.92E-12	**2.02**	1.79E-18	**2.26**	.	.	.	.
FOS	FBJ murine osteosarcoma viral oncogene homolog; transcription factor involved in cell proliferation, differentiation, survival, hypoxia and angiogenesis	1.4E-10	**1.98**	2.57E-16	**2.32**	.	.	.	.
C8orf4	Chromosome 8 open reading frame 4; uncharacterized protein	4.03E-14	**2.09**	2.74E-16	**2.21**	.	.	.	.
DUSP1	Dual specificity phosphatase 1; dephosphorylates MAP kinase MAPK1/ERK2	1.26E-18	**2.77**	9.3E-25	**3.19**	.	.	.	.
DUOX2	Dual oxidase 2; plays a role in antimicrobial defense at the mucosal surface	2.39E-12	**2**	3.64E-15	**2.01**	.	.	.	.
SLC6A14	Solute carrier family 6 (amino acid transporter), member 14; mediates the uptake of a broad range of amino acids	1.21E-17	**1.85**	3E-23	**2.15**	.	.	0.0000115	**1.14**
VNN1	Vanin 1; amidohydrolase recycling pantothenic acid (vitamin B5) and releasing cysteamine	3.36E-08	**-1.88**	2.24E-10	**-2.14**	0.000497	**-1.51**	0.0000133	**-1.82**
		0.0000101	**-1.87**	0.00000036	**-2.16**	0.0109	**-1.2**	0.000316	**-1.75**
		0.000102	**-1.62**	0.000000164	**-2.17**	.	.	.	.

Table 1: Differentially expressed genes in normal and colorectal tumor tissue. Gene expression was compared: pre, before hepatic pedicle clamping; post, after clamping; 10', 10 minutes after resection; 20', 20 minutes after resection, and 45', 45 minutes after resection.

Changes in gene expression due to ischemia (Affymetrix and qPCR)

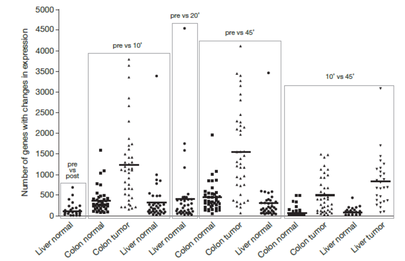

Figure 3: The variability of gene expression changes between patients, tissue type, surgery and tissue processing times. The figure shows the number of genes whose expression changed by more than 2-fold.

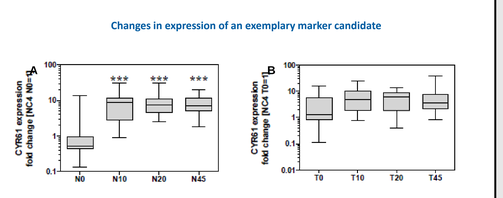

Figure 4: CYR61 expression in ischemic samples from normal tissue. Time dependent CYR61 expression in normal tissue is shown in A) and tumor tissue in B). Results are shown as fold changes normalized to sample NC4/N0 and NC4/T0. Kruskal-Wallis test and Dunn`s Multiple Comparison test was used for statistical analysis. *** p ≤ 0.001.

Changes in protein overall- and isoform-phosphorylation due to ischemia (Simple Western Charge – NanoPro™1000)

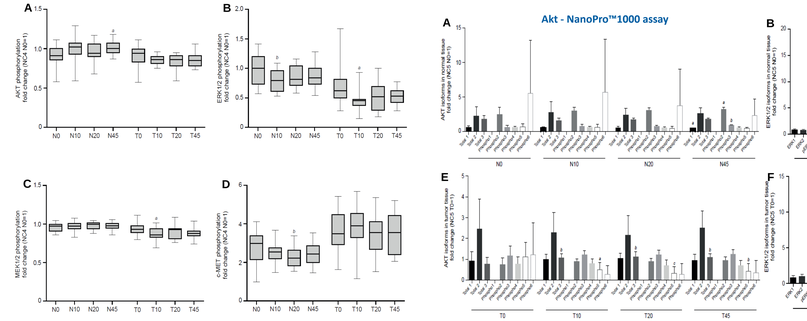

Figure 5: Time-dependent changes in overall phosphorylation of AKT (A), ERK1/2 (B), MEK1/2 (C), and c-MET (D) in normal (N) compared with tumor (T) Tissue from 20 patients are shown by box-and-whisker plots. Results were displayed as fold changes normalized in normal tissue to sample NC4/N0 and in tumor tissue to sample NC4/T0. Kruskal–Wallis test and Dunn test for multiple comparisons or analysis of variance (ANOVA) were used for statistical analysis. N = normal tissue; T = tumor tissue; 0 = before surgery; 10, 20, 45 = 10, 20, 45 min after resection; a=p≤0.05; b=p≤0.01.

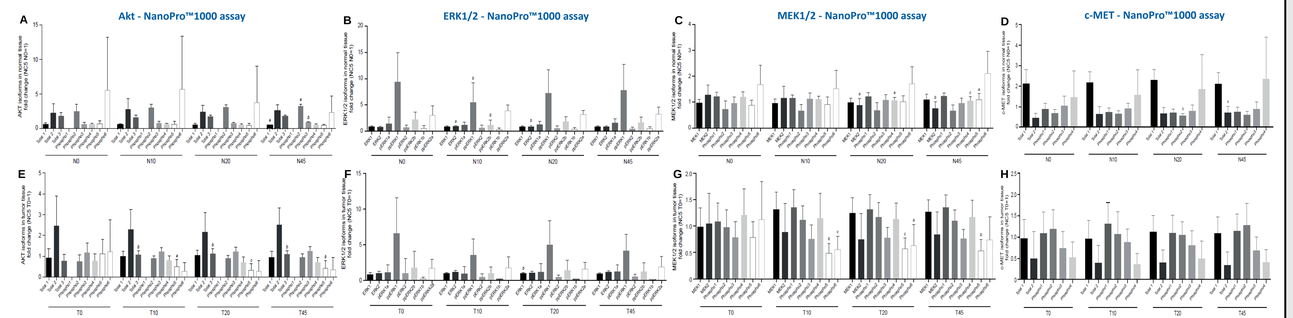

Figure 6: Changes in isoform phosphorylation of the target signaling proteins, AKT, ERK1/2, MEK1/2 and c-MET in response to ischemia. An overview of all isoforms of AKT, , ERK1/2, MEK1/2 and c-MET in normal tissue (A-D) of all patients analyzed is shown; therein results were displayed as fold changes normalized in normal tissue to sample NC5/N0 and in tumor tissue (E-H) to sample NC5/T0. An overview of all isoforms in tumor tissue of all patients analyzed is shown in below. Kruskal–Wallis test and Dunn test for multiple comparisons or analysis of variance (ANOVA) were used for statistical analysis. N = normal tissue; T = tumor tissue; 0 = before surgery; 10, 20, 45 = 10, 20, 45 min after resection; Time points (10, 20, 45) compared with time point 0; a= p≤0.05; b= p≤0.01; c= p≤0.001.

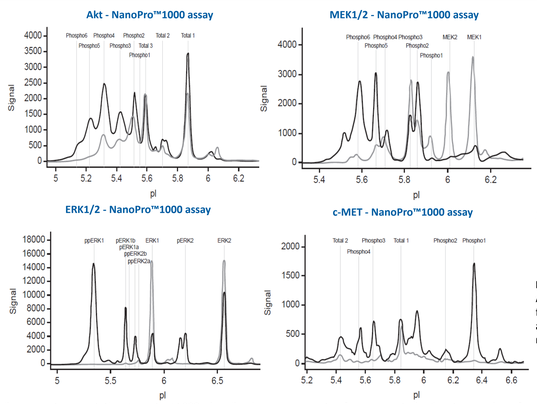

Figure 7: Representative NanoPro™1000 chemiluminescence spectra for run controls. Spectra overlay of positive (blue) and negative (green) run controls for AKT, ERK1/2, MEK1/2, and c-MET (A, C, E, and G) are shown in order to visualize regulation of phosphorylation.

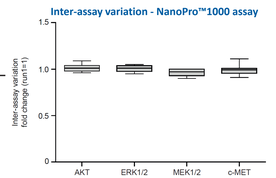

Figure 8: Inter-assay variation of NanoPro™1000 assays AKT, ERK1/2, MEK1/2, and c-MET. Data were calculated from run controls of all runs (n= 8). Boxes represent first and third quartile, whiskers represent minima and maxima, and solid lines within boxes indicate medians.

Immunohistochemistry

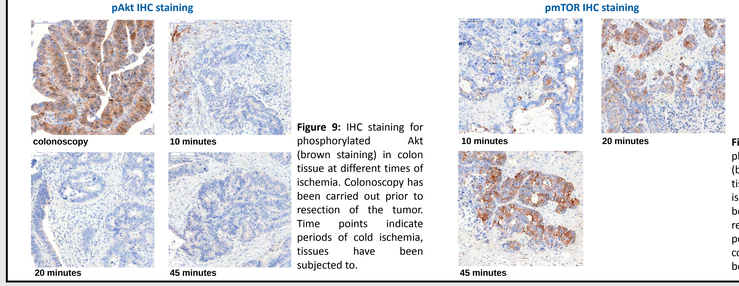

Figure 9: IHC staining for phosphorylated Akt (brown staining) in colon tissue at different times of ischemia. Colonoscopy has been carried out prior to resection of the tumor. Time points indicate periods of cold ischemia, tissues have been subjected to.

Figure 10: IHC staining for phosphorylated mTOR (brown staining) in colon tissue at different times of ischemia. Colonoscopy has been carried out prior to resection of the tumor. Time points indicate periods of cold ischemia, tissues have been subjected to.

Conclusion

An understanding of tissue data variability in relation to processing techniques during and postsurgery is mandatory when testing surgical specimens in the context of clinical diagnostics, drug development, or identification of predictive biomarkers. To obtain reliable expression data, tissue processing for research and diagnostic purposes needs to be highly standardized. Alterations in molecular patterns due to ischemia, identified in this study have to be analyzed with caution in research and development programs.