Quantitative analysis of gene expression in fixed colorectal carcinoma samples as a method for biomarker validation

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Abstract. Biomarkers have been described as the future of oncology. Modern proteomics provide an invaluable tool for the near-whole proteome screening for proteins expressed differently in neoplastic vs. healthy tissues. However, in order to select the most promising biomarkers, an independent method of validation is required. The aim of the current study was to propose a methodology for the validation of biomarkers. Due to material availability the majority of large scale biomarker studies are performed using formalin-fixed paraffin-embedded (FFPE) tissues, therefore these were selected for use in the current study. A total of 10 genes were selected from what have been previously described as the most promising candidate biomarkers, and the expression levels were analyzed with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using calibrator normalized relative quantification with the efficiency correction. For 6/10 analyzed genes, the results were consistent with the proteomic data; for the remaining four genes, the results were inconclusive. The upregulation of karyopherin α 2 (*KPNA2*) and chromosome segregation 1-like (*CSE1L*) in colorectal carcinoma, in addition to downregulation of chloride channel accessory 1 (*CLCA1*), fatty acid binding protein 1 (*FABP1*), sodium channel, voltage gated, type VII α subunit (*SCN7A*) and solute carrier family 26 (anion exchanger), member 3 (*SLC26A3*) was confirmed. With the combined use of proteomic and genetic tools, it was reported, for the first time to the best of our knowledge, that *SCN7A* was downregulated in colorectal carcinoma at mRNA and protein levels. It had been previously suggested that the remaining five genes served an important role in colorectal carcinogenesis, however the current study provided strong evidence to support their use as biomarkers. Thus, it was concluded that combination of RT-qPCR with proteomics offers a powerful methodology for biomarker identification, which can be used to analyze FFPE samples.

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Introduction

Neoplastic diseases are the second major cause of mortality in modern societies, and colorectal carcinoma is one of the most prevalent types (1). Biomarkers are prevalent and important in modern medicine. They enable earlier detection of cancer and aid in the prediction of prognosis. However, routine use of biomarkers remains low (2).

Proteomic technology can aid in more rapid progress in the search for biomarkers. However, despite more than 15 years of proteomic research, no effective novel biomarkers have been identified for colorectal carcinoma, hundreds of candidate biomarkers have been identified, however none are currently used in clinical practice (3,4). A key difficulty is the selection of biomarkers for further investigation; due to the fact that clinical trials for biomarker validation are expensive, it is important to select the most promising candidates. Unfortunately, in the majority of cases, promising proteins selected vary between studies, likely due to the differing protocols used. Quantitative data in proteomics are obtained in relation to external or internal standards, and since the standards used are different in each experiment, comparing the results is very difficult. Quantitative data has been previously published (5). Being aware of the controversies existing around the label-free proteomics methods (6), it was decided to validate the most promising biomarkers with a method widely recognized as being reliable in quantitative analysis, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method.

Formalin-fixed paraffin-embedded (FFPE) samples are widely available in large numbers, often with clinical and outcome information attached, making them ideal for biomarker studies. In the current study, routinely processed, archival FFPE samples were used. Although it is widely known that RNA isolated from FFPE tissue is highly degraded (7-11) it has become more commonly accepted that in spite of the poor RNA quality, gene expression

analysis from the fixed tissue is possible. According to the literature, use of short amplicons and normalization with more than one reference gene increases the accuracy of the measurements (11,12). It has also been previously identified that this approach can be applied to the analysis of colorectal carcinoma (10).

Materials and methods

Human FFPE specimens. Archival FFPE samples of cancer and adjacent normal colon tissue were obtained from the Pathology Department of Wrocław Medical University (Wrocław, Poland). The study was approved by the ethics committee of Wrocław Medical University. For the purpose of the investigation thirteen blocks containing tumor tissues and four blocks containing negative surgical margins (used as an approximation of healthy tissue) were selected. Detailed characteristics of the examined patients are provided in Table I.

RNA isolation. All work was conducted under conditions that minimized the exposure to RNases. The bench surface, all the equipment and the glass slides were cleaned with RNaseZap RNase Decontamination solution (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) prior to their use, according to the manufacturer's instructions. Diethyl pyrocarbonate (DEPC; Sigma-Aldrich, St. Louis, MO, USA)-treated water and hematoxylin (0.1%, v/v) were used throughout the histology procedures.

The FFPE blocks were cut using a standard microtome (Reichert-Jung Hn40; Leica Microsystems GmbH, Wetzlar, Germany) into 10-μm sections, which were then mounted onto glass slides (Superfrost; Menzel-Glaser; Thermo Fisher Scientific, Inc., Braunschweig, Germany). In each case, the first four sections were discarded to exclude any negative effects due to exposure to air. In order to avoid any contamination, a new microtome blade was used for each block.

All sections were incubated on a hotplate at 70˚C for 1 min and then were deparaffinized in two changes of xylene (Stanlab Sp. J., Lublin, Poland) for 3 and 2 min. Subsequently the samples were rinsed in alcohol (95% solution for 1 min and then 70% solution for 90 sec) and finally in DEPC water for 90 sec. To identify the tumor-enriched area, sections were stained with DEPC-treated hematoxylin solution (Sigma-Aldrich) for 20 sec and finally rinsed in DEPC-treated water for 1 min.

The cancer cells or healthy epithelium were scraped off the glass slides using sterile, RNaseZap-treated, single-use needle. Subsequently, they were transferred to nuclease-free 1.5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) and dried in 37˚C for 2 min. Microdissection was performed by the pathologist with microlance 3-26 G single-use needles (BD Biosciences, Franklin Lakes, NJ, USA) subsequent to microscopic examination of each slide with a Eclipse Ci microscope (Nikon Corporation, Tokyo, Japan). Approximately 1 cm^2 of tissue was scraped off for each isolation and two independent RNA isolations were performed for each FFPE block.

Total RNA was isolated with the High Pure RNA Paraffin kit (#03270289001; Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions, with certain modifications. Briefly, 100 μl tissue lysis buffer, 16 μl 10% sodium dodecyl sulfate and 40 μl proteinase K (20 mg/ml) from the kit were added to each sample, vortexed with a ZX3 Advanced Vortex Mixer (VELP Scientifica SRL, Usmate, Italy) and then incubated at 55˚C for 17 h. RNA was pooled by adding two lysates to each High Pure Filter tube. All purification, DNase treatment, subsequent digestion with proteinase K and all other steps were performed according to the manufacturer's protocol. RNA was then resuspended in 50 μl Elution buffer. Once the RNA was extracted, it was then DNase treated to remove any DNA contamination. DNA was eliminated using the RNase-Free DNase Set (Qiagen GmbH, Hilden, Germany) and the Rneasy Mini kit (Qiagen GmbH). DNase digestion was performed twice: First in the eluate and then on-column. All steps were performed according to the manufacturer's instructions.

The final volume of each sample was 30 μl. The purified total RNA was stored at -80˚C until used for cDNA synthesis.

Nucleic acid measurements. The concentration and the purity of the RNA were measured with the Picodrop Microliter UV/Vis Spectrophotometer (Picodrop, Ltd., Hinxton, UK). The purity of the RNA was determined by measuring the absorbance ratio A260/A280, with the ratio value ~2.0 being accepted as 'pure' RNA. The total amount of nucleic acids (RNA, DNA) and proteins was assessed by the Qubit 2.0 Fluorometer (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Qubit measurability limit for proteins is 1.0 μg/ml. The measurements of the RNA concentration were conducted using Picodrop and Qubit a minimum of 2 times for each sample, and the results are presented as the mean ± standard deviation. Prior to the RT-qPCR reaction, all the samples were applied to a concentration of 600 ng/μl.

The total RNA and mRNA integrity, purity and concentration were assessed by capillary electrophoresis using an Experion Bioanalyzer, Experion RNA HighSens Chips and Experion software, version 3.20 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's protocol.

cDNA synthesis. A reverse transcription reaction was performed in a 20 μl volume in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Quebec, Canada) using the Transcriptor First Strand cDNA Synthesis kit (#04379012001; Roche Diagnostics) according to the manufacturer's instructions, with certain modifications.

The components of the Transcriptor kit were used as follows: Total RNA (3 μl; 600 ng/μl), 50 pmol/μl Anchored Oligo $(dT)_{18}$ Primers (final concentration 2.5 μM) and 600 pmol/μl Random Hexamer Primers (final concentration, 60 μM) were heated in 7 μl RNase-free water at 65˚C for 10 min and immediately chilled on ice. A mixture consisting 4 μl Transcriptor RT Reaction buffer (final concentration of $MgCl_2$, 8 mM), 0.5 μl Protector RNase Inhibitor (40 U/μl; final concentration, 20 U), 2 μl 10 mM dNTP mix (final concentration 1 mM each for the four deoxyribonucleoside triphosphates; dATP, dCTP, dGTP and dTTP) and 0.5 μl Transcriptor Reverse Transcriptase (20 U/μl; final concentration, 10 U) were added to the RNA solution and incubated at 25˚C for 10 min, followed by 30 min at 55˚C. Finally,

Table I. Clinicopathological characteristics of study patients.

Patient	Gender/age	Grade	pTNM	Stage
1	M/78	G2	pT3N1Mx	III
2	M/76	G1	pT3N1Mx	III
3	M/89	G2	pT2N1Mx	III
4	M/62	G2	pT4N1Mx	III
5	F/84	G2	pT3N0Mx	II
6	M/69	G2	pT4N0Mx	II
7	M/77	G2	pT4N2M1	III
8	F/57	G2	pT2N2Mx	III
9	F/76	G2	pT3N0Mx	II
10	M/58	G3	pT2N1Mx	III
11	F/75	G2	pT2N2M1	IV
12	F/57	G2	pT2N2Mx	III
13	F/77	G2	pT1N2Mx	III

TNM, tumor, node, metastasis; M, male; F, female.

Transcriptor Reverse Transcriptase was inactivated by heating the reaction mixture to 85˚C for 5 min. Samples were immediately cooled on ice. The obtained cDNA (20 μl) was stored at -20˚C.

RT-qPCR. The RT-qPCR reaction was performed using the LightCycler 480 Instrument and RealTime Ready Custom panels (Roche Diagnostics), the ready to use LightCycler 480 Multiwell Plate 96 (Roche Diagnostics). Each well of the Multiwell Plate 96 contained both forward and reverse gene specific primers (8 pmol/primer) for the selected target and one Universal Probe Library probe (4 pmol), all pre-plated in the wells.

The total volume of the reaction was 20 μl. Each reaction mixture consisted of 3 μl reverse transcription reaction product as the template, 7 μl PCR grade water and 10 μl LightCycler 480 Probes Master (Roche Diagnostics) containing FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 6.4 mM $MgCl_2$ (all Roche Diagnostics). The RT-qPCR reaction was completed with an initial denaturation step (95˚C for 10 min) followed by 55 cycles of denaturation (95˚C, 10 sec), annealing (60˚C, 30 sec) and extension (72˚C, 1 sec). A final cooling step was conducted at 40˚C for 30 sec. Samples were analyzed in duplicates or triplicates.

A total of 10 genes from neoplastic tissues [karyopherin α 2 (*KPNA2*), chloride channel accessory 1 (*CLCA1*), transcription elongation factor A (SII) (*TCEA1*), G protein-coupled receptor, class C, group 5, member A (*GPRC5A*), paralemmin 3 (*PALM3*), chromosome segregation 1-like (*CSE1L*), fatty acid binding protein 1 (*FABP1*), high mobility group nucleosome-binding domain-containing protein 1 (*HMGN1*), sodium channel, voltage gated, type VII α subunit (*SCN7A*) and solute carrier family 26 (anion exchanger), member 3 (*SLC26A3*)] and 6 genes in the healthy tissues (*KPNA2*, *CLCA1*, *TCEA1*, *CSE1L*, *FABP1* and *HMGN1*) were examined. The following primer sequences were used: *KPNA2*, F 5'-GCATAAATAGCAGCAATGTGGA-3' and R 5'-GGGGCTGTTTTTCTCTGGA-3'; *CLCA1*, F 5'-TCATCAGGAAATGGAGCTGTC-3' and R 5'-CTGGCTGTTCTGGAGGGTTA-3'; *TCEA1*, F 5'-TTAAGGAAAAATGTCCTCTGTGG-3' and R 5'-GGTCAAGTTTTTCCGCATCT-3'; *GPRC5A*, F 5'-TCAAGAGGAAATCACTCAAGGTT-3' and R 5'-GTGGGATGGAGAATTCCTTTT-3'; *PALM3*, F 5'-GCACGTCCACCTAAACCTG-3' and R 5'-GGCTTCATCGCAGAAGGA-3'; *CSE1L*, F '-AGGTTATTGTGCCTAACATGGAA-3' and R 5'-TCCTCAGAATTATCTTCAAATGCTT-3'; *FABP1*, F 5'-GCAGAGCCAGGAAAACTTTG-3' and R 5'-CCTTCCCCTTCTGGATGAG-3'; *HMGN1*, F 5'-AGACTTACCTGCGGAAAACG-3' and R 5'-TGGCTTCTTTCTCTCCTGCTT-3'; *SCN7A*, F 5'-AAGGAGATTCAGAGTAAGTCTGGTG-3' and R 5'-CATTCAGATGAGCTAGATTGCTTT-3'; and *SLC26A3*, F 5'-ATTGTGGCGAAAGGACAAAT-3' and R 5'-ACTAGCTGCCAGGCCTAACC-3'. RT-qPCR was performed using calibrator normalized relative quantification with the efficiency correction. Peptidylprolyl isomerase A (cyclophilin A) (F TTCATCTGCACTGCCAAGAC and R CACTTTGCCAAACACCACAT) and β-actin (F TCCTCCCTGGAGAAGAGCTA and R CGTGGATGCCACAGGACT) were used as reference genes in the analysis. A mixture of RNA isolated from the healthy mucosa of four patients was used as a calibrator. The RT-qPCR efficiency differences were corrected with the standard curves made for each reference and each target gene. The negative controls were prepared by omitting the addition of the reverse transcriptase to the cDNA synthesis reaction. 'Pure' DNA samples were considered those that exhibited no growth following 55 cycles of RT-qPCR. The positive controls consisted of three assays, each one targeting different portions of the same transcript: At the 3'-end, in the middle and at the 5'-end. Obtained data was analyzed with the LightCycler 480 Multiple Plate Analysis Software (Roche Diagnostics).

Statistics. Statistical analysis was performed using the Mathematica program, version 7 (Wolfram Research Europe, Ltd., Witney, UK). The linear models were built using all potentially significant variables; the genes of which expression levels in cancer tissues were significantly different from the levels in healthy tissues. Subsequent to estimating the values of the coefficients, all the insignificant variables were removed and the process was repeated, until a consistent model with significant parameters was obtained.

Clustering of all samples was performed using Mathematica. The Euclidean distance functions (between the expression levels of either all 4 significant genes or the 2 most significant genes) were taken into account as a measure of similarity. A total of two clusters were required, and a Logit model was built using the maximum likelihood method of parameter estimation.

Results

A total of 10 proteins either up- or downregulated in cancer were selected from a previously published proteomic study (5). RT-qPCR was performed using calibrator normalized relative quantification with the efficiency correction. The complete

list of the analyzed genes/proteins with aliases and additional information including functional class, amplicon length, intron spanning and position and transcript length is provided in Table II. Short amplicons [67-113 base pairs (bps)] were used, which are preferable in the case of highly degraded RNA (12).

Capillary electrophoresis was performed for all samples and for the calibrator and the results are presented in Fig. 1. The concentration of the isolated RNA, calculated RNA quality indicator and the concentration of contaminating DNA and proteins is presented in Table III. Due to the fact that all negative controls exhibited no growth after 55 cycles, it was concluded that the concentration of contaminating DNA was too low to interfere with the RT-qPCR results. Negative controls for each sample were prepared by omitting the reverse transcription step, and running together with the samples.

In the current study, the expression levels of 10 genes in cancer vs. healthy tissues in samples obtained with needle microdissection from 13 patients were analyzed. The results are presented in Table IV.

Out of the 10 analyzed genes, 6 exhibited statistically significant different expression levels in the neoplastic vs. healthy mucosa. Out of 6 proteins upregulated according to a previous proteomic study (5), only in 2 cases was statistically significant upregulation in gene expression observed in the current study. By contrast, out of 4 downregulated proteins, statistically significant gene expression was observed in all cases. The results are presented in Table V.

For the 6 genes analyzed in healthy and neoplastic tissues, cluster analysis was conducted. When the four significantly changed genes (*KPNA2*, *CSE1L*, *CLCA1* and *FABP1*) were analyzed together, certain cancer samples clustered together with the healthy samples. However, if the two most significantly altered genes (*CLCA1* and *FABP1*) were considered alone, then all healthy samples were observed to be clustered together and were separate from the neoplastic samples. These observations were used to construct the logit model.

In addition, linear modeling was conducted for stage, and the tumor (T) and necrosis (N) features separately. It was identified that only the *CLCA1* and *FABP1* genes served significant roles in these models. It was identified that the variability of gene expression for *CLCA1* accounted for 68% of the variability of the cancer stage (coefficient of determination =68%). In addition, expression of *CLCA1* was observed to be reduced by 3.1 times, while the stage was increased by 1. Concerning T, the expression of one gene was observed to be statistically significant, *FABP1*, and its variability accounted for 61% of the variability of T. Expression of *FABP1* was reduced by 2.4 times as the T feature increased by 1. For the N feature, the expression of one gene was observed to be statistically significant, *CLCA1*. However, its variability accounted for 27% of the total variability, which was low compared with the 61% observed for T and the 68% for stage.

Discussion

The importance of *CSE1L* in colorectal carcinoma has been previously suggested in numerous studies, however its precise role remains to be fully elucidated (13-17). CSE1L was selected for inclusion in the current study as a positive control. Additionally, the aim of the current study was to investigate whether it was possible to analyze *CSE1L* expression in FFPE samples, due to the fact that the majority of previous studies utilized immunohistochemistry. CSE1L serves an important role in the mediation of apoptosis and invasion of neoplastic cells (13,17,18). Notably, an increase in the levels of CSE1L in the sera of patients with metastasis was also reported (16). *CSE1L* is located on chromosome 20q; and upregulation of this chromosome has been previously reported as a common event in colorectal carcinogenesis (19). It has been previously suggested that CSE1L expression correlates with tumor stage and prognosis, however currently available data remains inconclusive (14,16,17). Utilization of archival FFPE samples with survival data should allow for the analysis of CSE1L expression in larger cohorts leading to more reliable conclusions. The results of the current study indicate that in the majority of cases of colorectal carcinoma there is an overexpression of CSE1L. These results are in agreement with a previously published gene expression study utilizing fresh tissues (17). In the current study, it was identified that quantitative analysis of *CSE1L* expression with RT-qPCR from fixed samples is possible. Due to the limited number of patients included, the association between gene expression and stage cannot be confirmed.

The KPNA2 protein remains to be fully investigated, however a previous study suggested that it serves a near-universal role in carcinogenesis (20). It was recently suggested that KPNA2 regulates the subcellular localization of DNA damage response proteins (21), and correlates with the proliferation activity (22). Data on *KPNA2* expression in colorectal carcinoma remains limited, however a previous study indicated that KPNA2 is overexpressed in this malignancy (20).

As described above, a logit model was constructed on two genes, *CLCA1* and *FABP1*, of which the expression levels separated healthy samples from neoplastic samples in the cluster analysis. Due to the small sample size, the coefficients of the logit model were not observed to be significant. As there is limited existing data, expression analysis of *CLCA1* and *FABP1* allows the 'prediction' (via a logit model) of the status of tissues (cancer vs. healthy) with a probability close to 1. Thus, it may be the case that expression analysis of these two genes in larger groups is a promising direction for further studies.

The role of *CLCA1* in carcinogenesis remains to be fully elucidated, with only five reports investigating the significance of *CLCA1* in cancer progression (23-27) and three concerning colorectal neoplasia (23,26,27) identified in the search conducted. It has been suggested that *CLCA1* acts as a tumor suppressor gene by suppressing Bcl-2 overexpression in response to cell swelling (26). Notably, data from ovarian cancer analysis indicated that *CLCA1* was overexpressed during cancer progression (24). This is in contrast to the downregulation of *CLCA1* in colorectal carcinogenesis, reported in numerous previous studies (23,26,27). A paper published in 2015 reported that there was a correlation between poorer prognosis and lower expression levels of CLCA1 in colorectal carcinoma, assessed by immunohistochemistry (26). To the best of our knowledge, the current study is the first genetic study to indicate a negative correlation between the cancer stage and *CLCA1* expression.

A greater number of studies have been conducted investigating the significance of *FABP1* in carcinogenesis, than

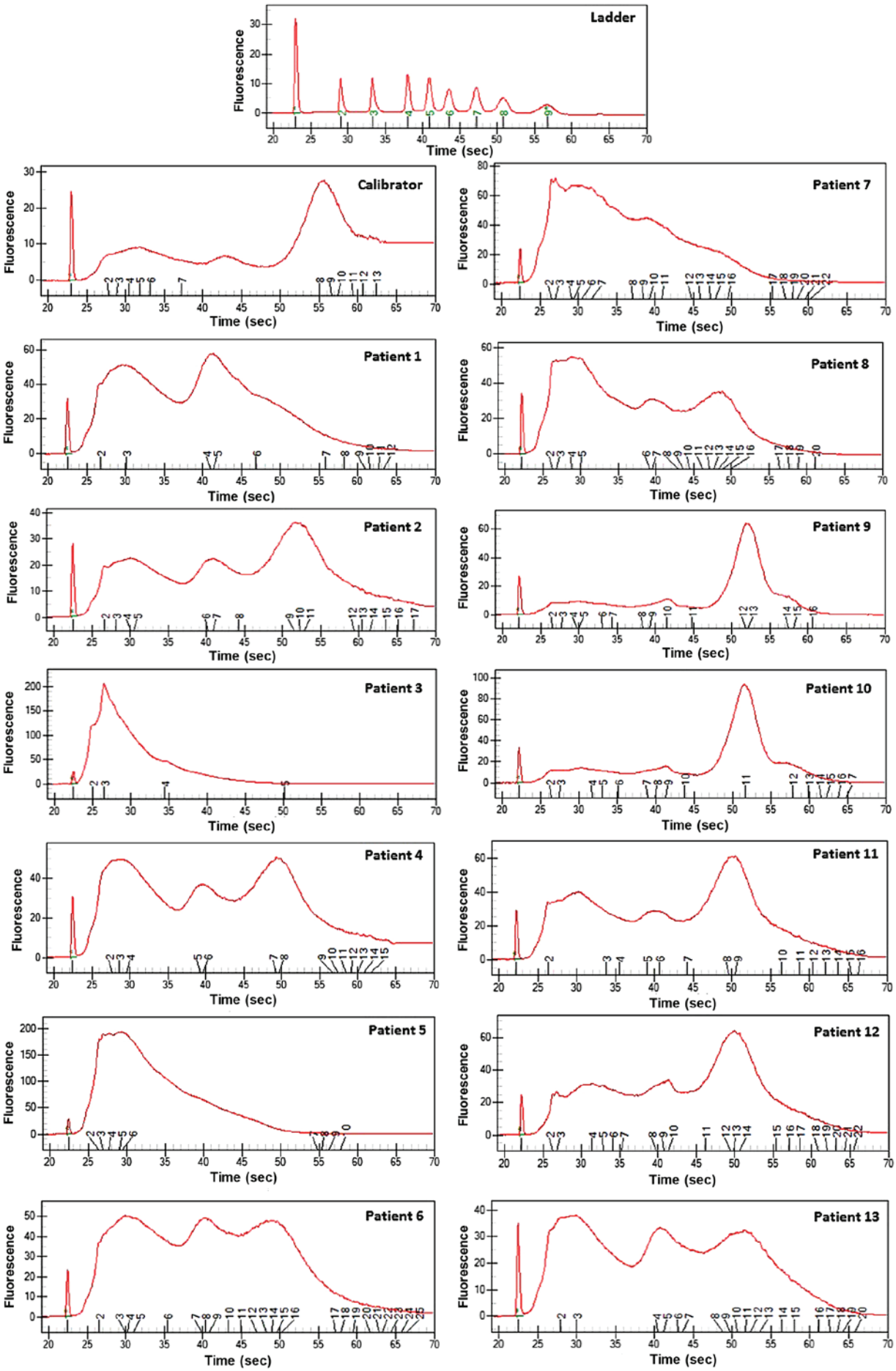

Figure 1. Capillary electrophoresis of all analyzed samples.

Table II. Characteristics of investigated genes.

Protein	Gene symbol	Amplicon length (bps)	Intron spanning and position	Transcript length (bps)
Nuclear transporters				
Importin subunit α2	*KPNA2*	81	Yes/435	1,979
Exportin 2	*CSE1L*	67	Yes/1,190	3,553
Non-nuclear channels and transporters				
Calcium-activated chloride channel regulator 1	*CLCA1*	75	Yes/1,594	3,118
Fatty acid-binding protein, liver	*FABP1*	74	Yes/167	546
Sodium channel protein type 7 subunit α	*SCN7A*	71	Yes/2,720	5,727
Chloride anion exchanger	*SLC26A3*	94	Yes/1,593	2,863
General transcription factors				
Transcription elongation factor S-II	*TCEA1*	108	Yes/1,002	1,226
High mobility group proteins				
High mobility group protein N1	*HMGN1*	77	Yes/459	1,303
Plasma membrane proteins				
Retinoicacid-induced protein 3	*GPRC5A*	113	Yes/1,573; 1,632	2,849
Paralemmin 3	*PALM3*	76	No	2,260

bps, base pairs.

Table III. RNA isolation results (formalin-fixed paraffin-embedded human colon).

Patient	Concentration (ng/μl)	RQI	gDNA (ng) in 100 ng RNA	Protein (μg/ml)
1	759	3.5	2.9	<1.0
2	520	3.5	9.3	<1.0
3	1101	Critical anomaly	2.1	<1.0
4	716	6.2	3.6	<1.0
5	1381	1.8	2.1	<1.0
6	763	3.5	2.3	<1.0
7	721	2.4	2.2	<1.0
8	645	3.1	2.5	<1.0
9	520	2.9	3.2	<1.0
10	838	2.5	2.4	<1.0
11	679	3.0	2.6	<1.0
12	651	2.5	2.8	<1.0
13	643	3.4	3.2	<1.0
Calibrator	652	3.7	4.1	<1.0

RQI calculated by Experion software. RQI, RNA quality indicator; gDNA, genomic DNA.

that for *CLCA1*. Previous studies (28-30) have reported the downregulation of FABP1 in colorectal carcinogenesis; with a correlation between poorer prognosis and lower expression levels of *FABP1* in liver metastasis also reported (31). An immunohistochemical study reported a correlation between tumor grade and FABP1 expression, however no correlation between stage and FABP1 expression was identified (29). To the best of our knowledge, the current study was the first to demonstrate an association between tumor size and FABP1 expression. Notably, although for hepatocellular carcinoma the association between FABP1 expression and prognosis is similar to that in colorectal carcinoma (32), in gastric carcinoma (33,34) and pancreatic carcinoma (35) the association was the opposite; FABP1 expression was observed to increase throughout cancer development.

SLC26A3 was previously identified as a potential tumor suppressor gene involved in colorectal carcinogenesis (36). Subsequent to the identification of an association between

Table IV. Gene expression ratios, cancer:normal (calibrator) for 13 individual patients.

	Patient												
Gene	1	2	3	4	5	6	7	8	9	10	11	12	13
KPNA2	9.89	3.53	8.73	12.8	17.83	3.36	11.55	7.73	2.16	6.65	3.14	4.58	9.59
CSE1L	13.7	10.68	2.5	4.27	0.73	2.42	3.85	2.07	4.46	3.51	1.88	2.1	6.71
CLCA1	0	$1.09x10^{-8}$	0.02	0	0	0	0.007	0.001	0	0	0.03	0.002	0.0001
FABP1	0.19	0.03	0.28	0.007	0.0009	0.01	0.05	0.3	0.06	0.002	0.03	0.40	0.05
SCN7A	0	0	0.06	0	0.02	0.28	0.1	0.27	0	0.008	0.17	0.03	0
SLC26A3	0	0.003	0.01	0	0.007	0.003	0.00007	0.008	0.001	0	0.0004	0.03	$2.52x10^{-8}$
TCEA1	2.89	0.78	0.48	1.35	0.59	1.78	2.05	0.9	0.33	0.78	0.5	0.75	1.4
HMGN1	8.5	0.69	0.62	1.8	0.35	0.83	0.5	0.59	0.59	0.83	0.88	0.62	1.3
GPRC5A	9.93	0.15	0.46	1.41	1.49	1.09	0.47	0.51	0.62	0.34	0.25	0.61	1.16
PALM3	8.61	0.07	4.45	0.27	0.34	163.5	2.04	0.81	0.19	0.13	10.44	1.68	10.67

Scores of 0 were considered as cancer samples with no growth after 55 cycles whereas the calibrator exhibited a constant Cp in all cases.

Table V. Gene expression ratios in comparison with proteomic data.

		Proteomic study (5)		Present study		
Protein	Gene	Average C/N ratio	P-value	Average C/N ratio	Standard deviation	P-value
Importin subunit α2	*KPNA2*	5.8	0.0083	7.8	4.6	**0.0002**
Exportin 2	*CSE1L*	8.8	0.0033	4.5	3.8	**0.007**
Calcium-activated chloride channel regulator 1	*CLCA1*	0.031	0.0010	0.0005	0.01	**$1.9x10^{-25}$**
Fatty acid-binding protein, liver	*FABP1*	0.16	0.0461	0.1	0.1	**$3.4x10^{-11}$**
Sodium channel protein type 7 subunit α	*SCN7A*	0.068	0.0186	0.07	0.1	**$7.7x10^{-13}$**
Chloride anion exchanger	*SLC26A3*	0.12	0.00708	0.006	0.01	**$2.3x10^{-25}$**
Transcription elongation factor S-II	*TCEA1*	4.5	0.0033	1.1	0.7	0.57
High mobility group protein N1	*HMGN1*	5.4	0.0047	1.4	2.2	0.54
Retinoicacid-induced protein 3	*GPRC5A*	131.0	0.0017	1.4	2.6	0.58
Paralemmin 3	*PALM3*	22.8	0.0049	15.63	44.6	0.28

Significant alterations in expression are indicated by P-values in bold. Data in columns 3 and 4 originate from previously published proteomic study (5). C, cancer; N, normal.

SLC26A3 and congenital chloride diarrhea (37), it was suggested as an electrolyte transporter. Previous studies have confirmed the downregulation of *SLC26A3* in colorectal carcinoma (38-40), and this has been explained to be as a result of dedifferentiation (41). Notably, it has been reported that *SLC26A*-deficient mice exhibit enhanced colonic crypt proliferation (42) and that *SLC26A3* overexpression can inhibit growth of cancer cell lines *in vitro* (43). Patients with germline mutations in *SLC26A3* have been demonstrated to exhibit a marginally increased risk of colorectal cancer (44). The data of the current study indicates that *SLC26A3* is downregulated in cancer, however this requires further investigation.

According to the UniProt database, *SCN7A* encodes for a voltage-dependent sodium channel of excitable membrane (45). No previous studies were identified that investigated the expression of *SCN7A* in colonic tissue, however data from The Human Protein Atlas confirmed its presence in healthy and neoplastic colon epithelium (46). The only identified study concerning *SCN7A* significance in carcinogenesis reports its DNA mutation in adrenocortical carcinoma (47). To the best of our knowledge, the current study together with a previous proteomic study (5) are the first to report downregulation of *SCN7A*, confirmed on genetic and proteomic levels.

For *TCEA1*, *HMGN1*, *GPCR5A* and *PALM3*, no statistically significant differences were observed. Although the results obtained with genetic methods are not contrary to those obtained with proteomic methods, there are considerable differences between the P-values obtained for each gene/protein. The absence of significance observed in the present study is suggested to be due to the extensive variability in the expression ratios. Due to the fact that little is known about *PALM3* and *HMGN1* significance in colorectal carcinogenesis, it is

difficult to identify which set of results are more reliable. In the case of *TCEA1*, there is some evidence supporting the hypothesis that it is overexpressed in cancer (48,49), however there are currently no studies investigating its expression in colorectal carcinoma. Previous studies (50,51) have identified the overexpression of *GPCR5A* in a large patient cohort. There are at least two possible explanations for the differences between the results obtained in the proteomic and transcriptomic studies regarding *TCEA1*, *HMGN1*, *GPCR5A* and *PALM3*. Firstly, it is possible that the mRNA level does not necessarily correlate with the protein level. Alternatively, the discrepancies may be due to technical reasons associated with RT-qPCR and the degradation of RNA. In this context it is notable that the amplicons used for *TCEA1* and *GPCR5A* were the longest used in the current study, at 108 and 113 bps, respectively.

In conclusion, the current study demonstrated that quantitative gene expression analysis from fixed material can be a valuable method used for the validation of potential biomarkers identified in other experiments, such as proteomic studies. However, this method may be suitable only for certain genes and requires careful experimental design and the use of shorter amplicons.

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