ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Molecular characterization of colorectal cancer: Insights from miRNA, mRNA, and protein analysis

Supplementary File

1. Western blotting: Electrophoresis and blotting

To check the primary antibody specificities using Western Blotting, the proteins, which were taken from cultured cells by our group, were denatured at 95°C for 5 min after mixing 3 volumes of lysate with one volume of 4× loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 4% SDS, 0.2% glycerol and 0.2% bromophenol blue) freshly supplemented with β-mercaptoethanol (5% v/v final concentration) (Sigma Aldrich, UK) and then chilled on ice for another 5 min prior to loading. The denatured protein was then separated on NuPAGE 4 – 12% Bis-Tris Mini Gels (Novex Life Technology, USA). The gel was placed into the electrophoresis tank (Invitrogen Life Technology, USA) containing 1× NuPAGE MOPS SDS running buffer (Novex Life Technology, USA).

The samples (15 μL), 3 μL of MagicMarkTM (Invitrogen life Technology) and 2 μL of Amersham rainbow marker (GE Healthcare, USA), were loaded on the gels and run at 125 V for 1.5 h. Afterward, the gel filter sandwich was prepared to transfer the separated proteins onto an Amersham nitrocellulose membrane (GE Healthcare) in NuPAGE transfer buffer (Novex Life Technology, USA) at 25 V for 30 min. The gel filter sandwich includes the gel, nitrocellulose membrane and filter papers. The gel was placed onto the filter paper, which was soaked in transfer buffer, then the membrane, which was wetted with methanol and washed with transfer buffer, was placed onto the gel and finally placed the remaining filter papers on the top of the membrane. The membrane was then put in the blocking buffer (consisting of 5% non-fat milk powder, 1× PBS and 0.1% Tween-20) to block the nitrocellulose membrane at room temperature for 1 h on the shaker. After that the blocked membrane was incubated with the diluted primary antibodies for overnight at 4°C with agitation.

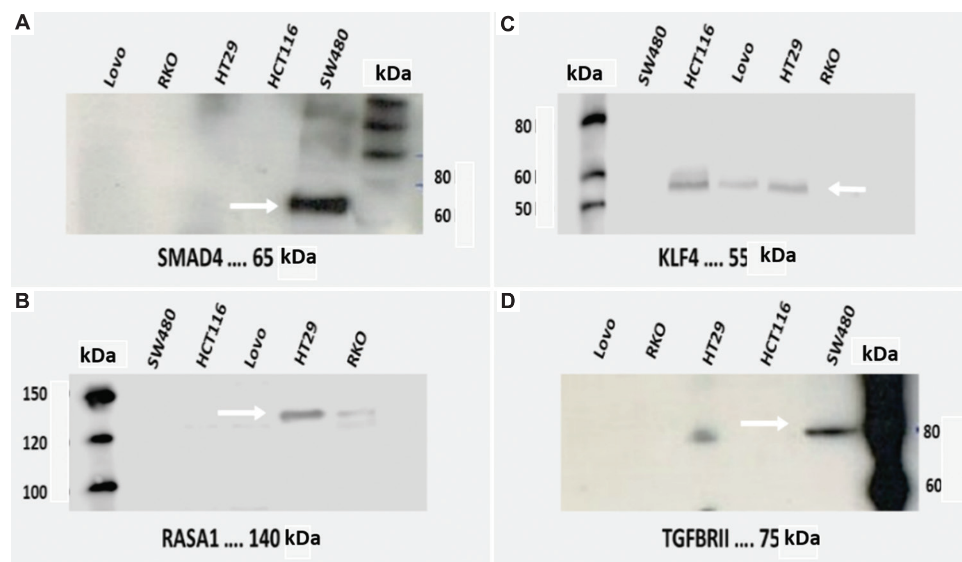

Figure S1. Expression of SMAD4, RASA1, KLF4, and TGFBRII in different cell lines. Lysates from human colon cancer were subjected to Western blotting to assess the expression of (A) SMAD4, (B) RASA1, (C) KLF4, and (D) TGFBRII. Different cell lines were used to check the specificity of the antibodies. Unfortunately, we did not obtain the expected bands for all antibodies in every cell line. However, we did observe specific bands for them in some cell lines. The lack of expected bands could be related to the expression levels of these proteins in the cell lines, potentially due to mutations and post-translational modifications of the proteins.

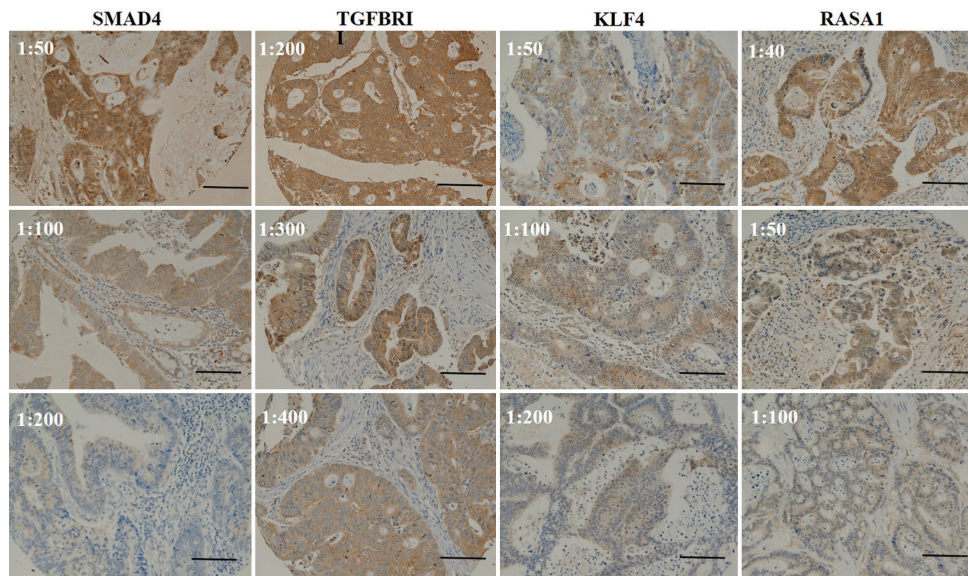

Figure S2. Optimization of anti-SMAD4, anti-TGFBRII, anti-KLF4, and anti-RASA1 antibodies. Different concentrations of each antibody were applied on colorectal tumor composite sections, and optimal concentration was chosen depending on heterogeneous staining pattern with no background staining (×200 magnification). The antibody dilution ratio shown on the top-left corner of each panel indicates the optimal antibody concentration that yields the optimal staining pattern. Scale bar: 100 μm.

Table S1. Clinicopathological features of patients

Variable	Classification	Frequency, *n* (%)
Dukes' stage	A	12 (15)
	B	37 (46)
	C	32 (39)
Vascular invasion	V0	40 (49)
	V1	40 (49)
	V2	1 (2)
Tumor grade	1	2 (3)
	2	74 (91)
	3	5 (6)
Tumor stage	pT1	3 (4)
	pT2	12 (15)
	pT3	46 (57)
	pT4	20 (24)

Table S2. Details of antibodies

Antibody	Size (kDa)	Cat no.	Type	Company
BCL2	25	Mo887	Mouse	Dako
SMAD4	65	Ab40759	Rabbit	Abcam
TGFBRII	75	Ab61213	Rabbit	Abcam
RASA1	140	Ab40677	Rabbit	Abcam
KLF4	50	12173S	Rabbit	Cell Signaling
PTEN	54	9554	Rabbit	Cell Signaling

Following this, three rounds of 5-min washes with PBS plus 0.1% Tween-20 were performed. After the last wash, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody solution (1:1000 diluted in blocking buffer; DAKO, Agilent Technology, USA), depending on species of the primary antibody (*i.e.*, polyclonal rabbit antimouse and polyclonal goat antirabbit; DAKO). Next, three washes of 5 min each were done with PBS plus 0.1% Tween-20. Nitrocellulose membrane was visualized by enhanced chemiluminescence (ECL) reagents (Supersignal West Pico Chemiluminescent Substrate, Thermo scientific, UK) and exposed to an Amersham hyperfilm ECL (Kodak, UK) for different periods to visualize any bands.

2. Optimization of primary antibodies for immunohistochemistry

Before conducting immunohistochemical staining, Western blotting analysis was applied to test the specificity of the primary antibodies. Those antibodies which did not give non-specific bands and specifically bound to the target proteins were chosen for immunohistochemical staining. Next, colorectal tumor composite tissue sections were used to determine optimal concentrations of primary antibodies using Novolink Polymer Detection Systems (Leica Microsystems). After testing different concentrations (higher and lower than the recommended one) of primary antibodies, the antibody concentration

Table S3. List of microRNA sequences of forward primers and the corresponding Qiagen catalog numbers

miRNA	Sequence of forward primer	Qiagen cat no.	Primer efficiency	Primer efficiency (%)
RNU6B	5'-ACGCAAATTCGTGAAGCGTT-3'	MS00033740	RNU6B	102.00
RNU61	5'-UCACCGGGUGUAAAUCAGCUUG-3'	MS00033705	RNU61	95.00
miR-20a-5p	5'-UAAAGUGCUUAUAGUGCAGGUAG-3'	MS00003199	miRNA20a	93.00
miR-21-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'	MS00009079	miRNA21	96.00
miR-29a-3p	5'-UAGCACCAUCUGAAAUCGGUUA-3'	MS00003262	miRNA29a	105.00
miR-31-5p	5'-AGGCAAGAUGCUGGCAUAGCU-3'	MS00003290	miRNA31	96.00
miR-92a-3p	5'-UAUUGCACUUGUCCCGGCCUGU-3'	MS00006594	miRNA92a	96.00
miR-224-5p	5'-UAUUGCACUUGUCCCGGCCUGU-3'	MS00003878	miRNA224	90.00

Table S4. NCBI reference sequence and qPCR primer sequences

Gene	NCBI Reference sequence	Sequence of primers	Source of primers	Primer efficiency (%)
SMAD4	NM_005359.5	Forward: 5'-GTGCATATATAAAGGTCTTTGAT-3'	Designed	96.00
		Reverse: 5'-GCTGACAGACTGATAGCTGGA-3'		
PTEN	NM_001304717	Forward: 5'-GGACCAGAGACAAAAAGGGAGT-3'	Designed	90.00
		Reverse: 5'-AGACCACAAACTGAGGATTGC-3'		
TGFBRII	NM_003242.5	Forward: 5'-CAGGTGGGAACTGCAAGATA-3'	Designed	92.00
		Reverse: 5'-TTCTCCCACTGCATTACAGC-3'		
BCL2	NM_000657.2	Forward: 5'-GCTGGGATGCCTTTGTGGAA-3'	Designed	93.00
		Reverse: 5'-ACTTCACTTGTGCCCAGAT-3'		
KLF4	NM_001314052.1	Forward: 5'-CCGCTCCATTACCAAGAGC-3'	Designed	96.00
		Reverse: 5'-TTTCTCACCTGTGTGGGTTC-3'		
RASA1	NM_002890.2	Forward: 5'-GGCCGGTATTATAACAGCATT-3'	Designed	92.00
		Reverse: 5'-TGTTCTTGATCCTGCATTGG-3'		
HPRT	NM_000194.2	Forward 5'-TGA GGC TCG CTT CTT GGA-3'	Designed	94.00
		Reverse 5'-GCT GAT GAC TGC TGG TCA-3'		

Abbreviation: NCBI: National Center for Biotechnology Information.

that demonstrated a heterogeneous staining pattern without background staining was picked as the best one for immunohistochemical staining.

3. Immunohistochemical staining

TMA slides were preheated at 60°C for 10 min to melt the paraffin and then left on the bench for 10 further minutes to cool down to room temperature before being loaded onto an Autostainer XL Staining System ST5010 (Leica Microsystems, USA) for dewaxing and rehydrating. The slides were dewaxed in two xylene baths for 5 min each and then rehydrated in a sequence of methylated spirit industrial (IMS 0.89 S.G. 74 O.P., Fisher Scientific, USA) concentrations each for 2 min followed by washing

with water for 5 min. Once this has finished, the antigen retrieval was performed by placing the slides in either 10 mM sodium citrate buffer (pH 6.0) or EDTA (pH 9.0) and boiling the slides in a microwave at 750 W for 20 min. Sections were placed under running tap water to gently bring their temperature down to room temperature. Following antigen retrieval, sections were loaded into Shandon Sequenza coverplates (Thermo Fisher Scientific). To exclude any leak or possibility of presence of air bubbles, the coverplate was filled with pH 7.6 Tris-buffered saline (TBS). Next, slides were loaded with 100 µL of peroxidase block for 5 min and washed twice with TBS each for 5 min. After washing, the slides were incubated with 100 µL of protein block for 5 min and washed twice with TBS each for 5 min before incubating them for 1 h with primary antibody, which was diluted to optimal staining conditions using Leica Antibody Diluent (Leica Microsystems, USA), and washed twice with TBS each for 5 min. The slides were loaded with post primary block for 30 min and then washed twice with TBS each for 5 min, followed by Novolink Polymer (Leica Microsystems, USA) for 30 min and twice with TBS each for 5 min.

The slides were loaded with diaminobenzidine (DAB) for 5 min (DAB chormogen and DAB substrate buffer in a ratio of 1:20) and washed twice with TBS each for 5 min before loading with counter staining hematoxylin for 6 min and then washed twice with TBS each for 5 min. Finally, the slides were dehydrated in a series IMS (IMS 0.89 S.G. 74 O.P., Fisher Scientific) solutions and followed by two baths of xylene solutions for 5 min each and mounted with dibutylphthalate polystyrene xylene.

4. Assessment of protein expression

Initially, the stained TMA slides were checked with the light microscope to confirm the validity and staining, followed by slide scanning using a Nanozoomer Digital Pathology scanner (Hamamatsu Photonics, Japan) at ×20 magnification. A semi-quantitative method (H-score) was used to assess protein expression in tumor cells. In the H-scoring method, presence and intensity of immunoreactivity were assessed. Staining intensity of each core was assessed: 0 as negative, 1 as weak, 2 as moderate, and 3 as strong staining, and then H-scores were calculated by multiplying the percentage of positive tumor cells (minimum 0 and maximum 100) by the staining intensity [373]. After that, to ensure reproducibility, the slides were all assessed by a second scorer (Dr. Karzan Marf Murad), and intraclass correlation coefficient was applied to assess concordance between both scorers.