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Discovery of novel plasma proteins as biomarkers for the development of incisional hernias after midline incision in colorectal cancer patients: the ColoCare Study

Jürgen Böhm[1,*], **Frank Pianka**[2,*], **Nina Stüttgen**[2], **Junghyun Rho**[4], **Biljana Gigic**[2], **Yuzheng Zhang**[4], **Nina Habermann**[5], **Petra Schrotz-King**[3], **Clare Abbenhardt-Martin**, **Lin Zielske**[3], **Paul D. Lampe**[4], **Alexis Ulrich**[2], **Markus Diener**[2], and **Cornelia M. Ulrich**[1]

[1]Department of Population Health Sciences, Huntsman Cancer Institute and University of Utah School of Medicine, Salt Lake City, USA

[2]Department of General, Visceral and Transplantation Surgery, University of Heidelberg, Heidelberg, Germany

[3]Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Heidelberg, Germany

[4]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, USA

[5]Genome Biology, European Molecular Biology Laboratory, Heidelberg, Germany

Abstract

Background—Ventral incisional hernia is the most common long-term complication after abdominal surgery. Among newly-diagnosed colorectal cancer patients, we screened the pre-surgical plasma proteome to explore predictive markers for the development of an incisional hernia.

Methods—We utilized pre-operative plasma samples of 72 newly diagnosed colorectal cancer patients who underwent midline incision for tumor resection between 2010 and 2013. 21 patients with incisional hernia occurrence were matched with 51 patients with at least 18 months follow-up without an incisional hernia by gender, age, and BMI. To assess predictive markers of incisional hernia risk we screened the plasma proteome for >2,000 distinct proteins using a well-validated antibody microarray test. Paired t-tests were used to compare protein levels between cases and controls. A gene-set-enrichment analysis (Gene Ontology and KEGG) was applied to test for differences in signaling pathways between the two groups.

Results—The proteome screen identified 25 proteins that showed elevated or reduced plasma levels in the hernia group compared to the control group (nominal p-values <0.05). Several

Cornelia M. Ulrich, PhD, Population Sciences, Huntsman Cancer Institute, 2000 Circle of Hope, Rm 4165, Salt Lake City, Utah 84112-5550, 801-213-5716 Office, 801 587-4285 Assistant, Jessica Baker, 801-585-0900 Fax, neli.ulrich@hci.utah.edu.
*These authors contributed equally to this work.

proteins were in pathways associated with wound healing (CCL21, SHBG, BRF2) or cell adhesion (PCDH15, CDH3, EPCAM).

Conclusion—Our study shows that there are multiple individual and groups of plasma proteins that could feasibly predict the personal hernia risk prior to undergoing surgery. Further investigations in larger independent sample sets are warranted to replicate findings and validate clinical utility of potential biomarkers. After validation, such a biomarker could be incorporated into a multifactorial risk model to guide clinical decision making.

Introduction

Ventral incisional hernia is the most common long-term complication after laparotomy and is a continuing problem for the abdominal surgeon. By definition, hernias develop due to a defect in an aponeurotic tissue layer, e.g. at the incisional site following abdominal surgery, leading to the protrusion of an organ out of its natural cavity.[1] Incisional hernias occur more frequently after midline incisions compared with a transverse incisional approach.[2] Incisional hernia incidence after elective midline incision, e.g., for the oncologic resection of colorectal cancer, is high with reported 10–40%.[3, 4] Most incisional hernias develop within 2 years after surgery[5] and can cause severe health and cosmetic problems. Each year, over 340,000 hernia repairs are performed in the United States, causing health care costs of at least $3.2 billion.[6] These figures illustrate the tremendous economic burden associated with the incidence and repair of incisional hernia, and, consequently, demonstrate the importance of a multidirectional approach in the prevention of this complication. Preventive measures are available, such as prophylactic mesh implantation, leading to a reduction of an incisional hernia within 2 years after surgery.[7] However, after analysis of the existing evidence, the European Hernia Society concludes “Although the data are favorable and consistent for prophylactic mesh augmentation, the Guidelines Development Group decided that larger trials are needed to make a strong recommendation to perform prophylactic mesh augmentation for all patients within certain risk groups.”[8]

Not all patients experience the same risk of developing an incisional hernia, and the pathogenic mechanism of incisional hernia development is not fully understood. The genesis of an incisional hernia is multifactorial: Generally, conditions that negatively affect wound healing make patients susceptible to incisional hernia. Contributing factors may be divided into patient- and surgeon-related factors, which are to some extent directly controllable.[1, 9–15] Surgery-related factors include suture material, poor technique, and the need for emergency surgery.[16] Furthermore, postoperative wound infection at the surgical site has been identified as a major contributing factor, with up to 25 percent of patients developing an incisional hernia.[17, 18] Patient-related factors that contribute to the development of incisional hernia include, among others, age, obesity, smoking, malnutrition, immunosuppressive therapy, and connective tissue disorders.[19] Moreover, a positive family history of incisional hernia contributes to an elevated risk.[20] These findings support the hypothesis that certain individual biological factors may significantly increase the risk for incisional hernia development. Alterations in connective tissue metabolism such as changes in the extracellular matrix have been reported, including thinner collagen fibrils, imbalance between type I and type III collagen, and increased activity of collagen degrading matrix

metalloproteinases (MMPs).[21, 22] A decreased ratio of type I to III collagen and MMP-1/MMP-2 ratios in fascia tissue[23] and a systemic alteration of collagen metabolism[24] have been reported in hernia patients that might explain biological activities of key elements in the development of incisional hernia.

A predictive tool to precisely identify patients that are at high-risk for an incisional hernia is an unmet clinical need. A validated risk model shows predictive value for abdominal wound dehiscence[16] and a predictive model for estimating the risk of early incisional hernia has been developed.[25] However, no presurgical biomarker exists to date that predicts incisional hernia development prior to planned surgery. Such a biomarker, alone or incorporated into a multifactorial model, could guide decision making for early preventive measures (e.g., mesh implantation at primary surgery) and personalized recommendations for patients at risk.

Thus, we tested an array-based strategy for the discovery of predictive biomarker candidates among newly-diagnosed colorectal cancer patients undergoing median laparotomy using presurgical plasma samples while accounting for known clinical risk factors.

Patients and methods

Patients

This pilot case-control study is nested in the Heidelberg (Germany) site of the ColoCare Consortium. ColoCare is an international, multicenter, prospective cohort, with additional US sites at the Fred Hutchinson Cancer Research Center, Seattle (Washington), H. Lee Moffitt Cancer Center and Research Institute, Tampa (Florida) and Huntsman Cancer Institute, Salt Lake City (Utah), recruiting newly-diagnosed colorectal cancer patients (ICD-10 C18–C20) of all stages with the goal to investigate predictors of cancer recurrence, survival, treatment toxicities and health-related quality of life (ClinicalTrials.gov: NCT02328677).[26–28]

All ColoCare patients included in this study underwent clinically indicated midline incision along the linea alba for colorectal cancer removal at a single institution (Department of General, Visceral and Transplantation Surgery, University Hospital of Heidelberg) between October 2010 and January 2013.

Out of n=265 patients enrolled in ColoCare, n=72 patients were included in the study who met the following inclusion criteria: midline incision, presurgical blood, no mesh implantation at the primary surgery, no burst abdomen in the control group, complete clinical data on risk factors, follow-up >18 months, and a phone interview to confirm presence or absence of incisional hernia. A study flow diagram is presented in Figure 1. Characteristics of the n=193 patients excluded from the study were similar to the patients included in the study, except that the included patients were an average of 5 years younger (see Supplementary Table 1). Of the n=72 included patients, n=21 had developed an incisional hernia (=cases) and were matched to the n=51 patients without hernia (=controls). A step-wise matching procedure was performed. In a first matching step we included all variables of interest: gender, age, BMI, smoking, adjuvant chemotherapy, impaired wound healing and diabetes. In the proceeding matching steps variables were dropped

consecutively. Patients were matched by at least gender, age and BMI. Table 1 shows patient characteristics abstracted from patient's medical records.

The study was approved by the Institutional Review Board of the Medical Faculty at the University of Heidelberg and all participants provided written informed consent.

Definition of incisional hernia cases and controls

The incidence of an incisional hernia at the midline incision site was assessed by a dual assessment of questionnaires and phone interviews. As a first step, we queried all patients for incisional hernia occurrences (along with a lay explanation and graphical illustration) in questionnaires at 6, 12, and 24 months post-surgery (see Supplementary Table 2). Second, we performed a comprehensive phone interview with the patient to verify whether an incisional hernia had occurred or not (see Supplementary Table 3). If a patient was not available by phone or the presence of incisional hernia remained unclear, the interview was conducted with the corresponding general practitioner. All incisional hernia cases reported the confirmation of hernia as part of a study-unrelated physical examination (e.g., with their general practitioner) or a surgical hernia repair. In addition, some cases reported confirmation by sonography (n=6) and internal CT scans were evaluated that confirmed hernia formation in n=3 cases. Patients were defined as controls if no incisional hernia had occurred within at least 18 months post-surgery, which was the time of the latest case of incisional hernia reported in the case group.

Plasma processing

Blood samples (EDTA) were collected from patients prior to surgery. Preparation of plasma was performed within 4 hours after blood-draw by retaining the supernatant after centrifugation (2500 g; 15 min) and storing in aliquots at −80 °C until analysis. 50 μl of each patient's plasma were shipped on dry ice to the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, Washington) for the antibody microarray experiments.

Antibody Microarray Experiments

Antibody microarray experiments were performed in the laboratory of Dr. Paul Lampe at FHCRC according to previously established methods.[29–31] Briefly, antibodies were printed in triplicates on Nexterion H hydrogel slides (Schott, Mainz, Germany) in 48 blocks with a 15×15 block format for a total of 3,600 unique features at a final concentration of 275 μg/ml. After depletion of albumin and IgG, the remaining plasma proteins (200 μg) were labeled with Cy5 (cases and controls) or Cy3 (reference) (GE Health Biosciences, Pittsburgh, PA) following a "case/control versus reference" procedure to remove dye bias from the analysis. After plasma was incubated on arrays, arrays were washed and scanned using an Axon GenePix 4200A microarray scanner (Molecular Devices, Sunnyvale, CA). GenePix Pro 6.0 image analysis software was used to analyze the scanned array images.

Statistical analysis

Detailed descriptions of array statistical analysis have been reported previously.[32] Briefly, for each antibody feature, the fold change of case and control signal (red channel) relative to the reference (green channel) was calculated as $\log_2(R_c/G_c)$, where R_c is red corrected and

G_C is green corrected applying a normexp background correction method.[33] Paired t-test was performed to assess the difference between case and control signal for each antibody. The p-values calculated via the paired t-test represent the ability of the protein marker to distinguish cases from controls. Protein markers were then ranked based on the coefficient, also known as the odds ratio, which is a $\log_2$-based measurement of signal intensity and represents a 2-fold change. Markers with positive coefficients are greater in cases versus controls, and negative coefficients represent the converse.

Gene Set Enrichment Analysis (GSEA) was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) gene sets that are available from the Molecular Signatures Database (MSigDB) (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The 889 antibodies available for analysis correspond to 732 unique genes. Of the 990 GO gene sets analyzed, our array included at least 5 gene members in 835 of these gene sets. With respect to KEGG gene sets, our arrays included at least 5 proteins corresponding to gene members in 129 of the 131 gene sets available. We then tested whether the proteins corresponding to groups of genes in a given gene set had a higher statistical ranking than the proteins not in this gene set based on Wilcoxon testing.

R statistical computing software program was used for all statistical array analyses incorporating the “limma” package for microarray read-in and normalization.[34]

Matching of cases and controls and the analyses of the study population’s characteristics were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Comparison of quantitative variables was performed by parametric Student’s t-tests. Pearson $\chi 2$ tests were used to investigate differences between categorical variables.

Results

The case and control group were balanced with respect to age, gender, BMI, tumor location and stage. Somewhat higher proportions of postoperative wound disorder, diabetes, and a history of other type of hernia (e.g., inguinal hernia) were found in the incisional hernia case group. Most incisional hernias (65%) occurred within the first 12 months post-surgery. The study population’s demographic and clinical characteristics are shown in Table 1.

Of the 3,290 proteins assessed in the paired t-test, 25 (0.8%) showed statistically significant differences in signal between cases and controls at $p<0.05$ with a reasonable effect size and $\log_2$ ratio of >1 or <−1. Of these 25 proteins, 12 were lower and 13 higher in cases compared to controls (Table 2). The top half of proteins that were lower in cases than in controls included proteasome subunit beta type 5 (− 1.52 $\log_2$ ratio), sex hormone-binding protein (−1.49 $\log_2$ ratio), defensin alpha 1 (−1.34 $\log_2$ ratio), chemokine (C-C motif) ligand 21 (−1.3 $\log_2$ ratio), SRA (steroid receptor RNA activator) stem-loop interacting RNA binding protein (−1.22 $\log_2$ ratio), and epithelial cell adhesion molecule (−1.13 $\log_2$ ratio). The top half of proteins that were higher in cases than in controls included RNA polymerase III transcription initiation factor (1.78 $\log_2$ ratio), calreticulin 3 (1.36 $\log_2$ ratio), estrogen receptor 1 (1.32 $\log_2$ ratio), harvey rat sarcoma viral oncogene homolog (1.2 $\log_2$ ratio), and fibronectin 1 (1.18 $\log_2$ ratio). A lower effect size of >0.5 or <−0.5 with p-values <0.05

revealed an additional 62 proteins that were different between cases and controls (Table 3), including Collagen Type I Alpha 1 (0.82 $\log_2$ ratio), Interleukin 1 Beta (0.78 $\log_2$ ratio), Fibrillin 2 (0.77 $\log_2$ ratio), Granulin (−0.51 $\log_2$ ratio), Nidogen 1 (− 0.63 $\log_2$ ratio), and Collagen Type XXIV Alpha 1 (−0.90 $\log_2$ ratio).

In our gene set analysis, a total of 11 KEGG and a total of 123 GO gene sets had a p-value <0.05 (Table 4). These pathways include ECM receptor interaction (p=0.0002), intestinal immune network for IgA production (p=0.0024), progesterone mediated oocyte maturation (p=0.0062), melanoma (p=0.0073), bladder cancer (p=0.0084), complement and coagulation cascades (p=0.0112), ubiquitin mediated proteolysis (p=0.0175), focal adhesion (p=0.0224), regulation of actin cytoskeleton (p=0.0267), pathways in cancer (p=0.0272), and toll-like receptor signaling pathway (p=0.0005) (Table 4).

Results were consistent when removing n=9 patients (n=4 cases; n=5 controls) with a previous inguinal or umbilical hernia from the analysis.

Discussion

Given the tremendous economic and patient burden, precision prevention of incisional hernias is an unmet clinical need. A precise risk prediction would allow personalized surgical measures in high-risk patients (e.g., preventive mesh implantation during the primary surgery) and individual patient recommendations to prevent the development of an incisional hernia. Certain risk factors have been identified. However, to date, no predictive pre-surgery blood-based biomarker is available to evaluate a patient's individual risk for an incisional hernia.

In presurgical plasma of colorectal cancer patients, we identified 25 proteins that were either nominally significantly elevated (13) or reduced (12) with a reasonable effect size ($\log_2$ ratio > 1 or < −1) in future incisional hernia patients. Most of these proteins are connected to cell adhesion, wound healing and inflammation. The cell adhesion molecules protocadherin-15, P-cadherin, and epithelial cell adhesion molecule (EPCAM) were decreased in future incisional hernia patients, and have previously been associated with wound healing and tissue integrity.[35–37] Furthermore, we observed decreased plasma levels of CC-chemokine ligand 21, a chemokine involved in inflammatory processes, in hernia cases compared to controls. Intradermal injection of CC-chemokine ligand 21 has been shown to increase the migration of mesenchymal stem cells resulting in accelerated wound repair in mice.[38] Moreover, sex hormone-binding globulin (SHBG) was decreased in future incisional hernia cases by a $\log_2$ ratio of −1.49 compared to controls, while estrogen receptor 1 was increased ($\log_2$ ratio of 1.32). Topical estrogen application has been shown to support wound healing in a randomized controlled trial[39] and elevated estrogen receptor 1 plasma levels may reflect receptor upregulation due to decreased estrogen binding by SHBG. RNA polymerase III transcription initiation factor (BRF2) was elevated in cases compared to controls ($\log_2$ ratio of 1.32). Overexpression of BRF2 has been associated with abnormal expression of E-cadherin and N-cadherin, marker proteins of the epithelial-mesenchymal transition (EMT).[40] Interestingly, proteasome subunit beta type-5 was decreased most ($\log_2$ ratio of −1.52) in future incisional hernia patients. As part of the 20S proteasome complex, it is responsible for

recognizing damaged proteins for protein quality control and proteasome inhibition, and has been associated with decreased proliferation of lens epithelial cells.[41] Finally, we discovered several proteins that differ between cases and control, for which no association with wound healing or connective tissue pathophysiology has been described in the literature thus far. GSEA analysis revealed sets or families of proteins that were changed in cases compared to controls. Based on this approach, pathways of interest included ECM (extracellular matrix) receptor interaction, focal adhesion, melanoma, bladder and pathways of cancer (Table 4).

One limitation of this study is that incisional hernias were assessed by a questionnaire and a phone interview, as physical examination by an experienced surgeon and imaging was not part of the ColoCare study design. Hence, small asymptomatic hernias may have been missed and misdiagnosis of a midline diastasis as a hernia may have occurred. Thus, our study reflects clinically apparent incisional hernias, which should be more important for clinical management. However, in future studies, we suggest to perform prospective study-related physical examination and respective imaging at pre-defined time points for the assessment of incisional hernia.

Another limitation is that our study population was restricted to colorectal cancer patients only. By our stringent inclusion criteria our case-control groups were very homogenous in their risk of developing an incisional hernia. However, we suggest in future directions that studies should not be limited to a specific disease group to identify a robust biomarker independent of clinical or demographic parameters.

A further limitation is that the medical chart reviews to collect data on clinical parameters were done retrospectively.

A limitation of the experimental array approach used is that we were only able to evaluate biomarkers for which antibodies were included on the array. Hence, no fully comprehensive assessment of the plasma proteome was performed and the potential of biomarkers not included on the array could not be assessed. This in particular limited our gene set analyses as we were limited by the candidates included on the array. However, the array covered a wide range of possible biomarker candidates yielding data on 3,290 proteins. The interpretation of the results is challenging given the study’s inherent pilot discovery nature. Nonetheless, this study shows proof-of-principle for array-based discovery of unique predictive biomarkers specific to incisional hernia and points to several proteins and pathways worth of further investigation.

In this pilot study, we applied, for the first time, an antibody microarray to compare the plasma proteome of future incisional hernia patients with matched clinical controls. We showed proof-of-principle for the discovery of novel plasma based biomarkers for incisional hernia occurrence by utilizing presurgical plasma samples. Our study shows that there are multiple individual and groups of plasma proteins that could predict the individual hernia risk prior to undergoing surgery. We were able to identify several protein biomarkers with a known association with wound healing or connective tissue pathophysiology. Promising biomarkers warrant further investigations in larger independent sample sets to further

characterize and validate their potential clinical utility. After validation, such a biomarker could be incorporated into a multifactorial risk model to guide clinical decision making.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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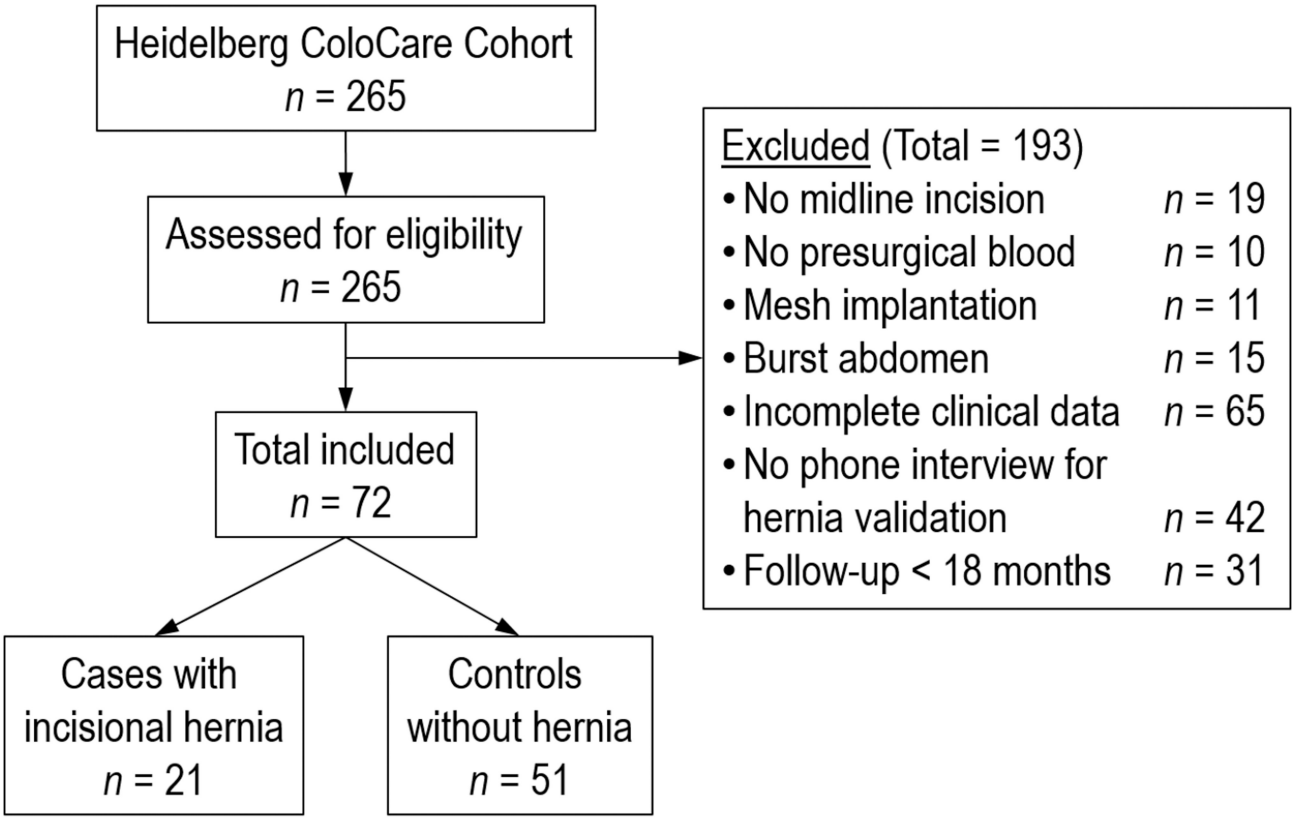

Figure 1.
Study flow diagram.

Table 1

Patient characteristics of incisional hernia cases and controls.

Variable	Cases with incisional hernia (*n* = 21)	Controls withou hernia (*n* = 51)
Age, *mean ± SD*	59.8 ± 11.4	58.9 ± 11.7
Gender, *n (%)*		
Male	16 (76)	30 (59)
Female	5 (24)	21 (41)
BMI (kg/m^2), *mean ± SD*	28.0 ± 4.6	26.3 ± 3.6
Tumor location, *n (%)*		
C18/C19 Colon or rectosigmoid	10 (48)	23 (45)
C20 Rectum	11 (52)	28 (55)
Stage, *n (%)*		
0/I/II	15 (71)	32 (63)
III/IV	6 (29)	19 (37)
Adjuvant therapy, *n (%)*	10 (48)	26 (51)
Neoadjuvant therapy, *n (%)*	6 (29)	19 (37)
Smoker, *n (%)*		
Current smoker	2 (9)	10 (20)
Ex-Smoker	1 (5)	4 (8)
Never smoker	18 (86)	37 (72)
Postoperative wound disorder, *n (%)*	5 (24)	4 (8)
Previous surgeries, *n (%)*		
Appendectomy	4 (19)	8 (16)
Hysterectomy	0 (0)	1 (2)
Ostomy	1 (5)	2 (4)
Cholecystectomy	2 (10)	2 (4)
Sterilization (male)	0 (0)	1 (2)
Sterilization (female)	1 (5)	0 (0)
Prostatectomy	1 (5)	0 (0)
Nephrectomy	0 (0)	1 (2)
Umbilical herniotomy	1 (5)	0 (0)
Caesarean section	0 (0)	2 (4)
Suture technique, *n (%)*		
Continuous	16 (76)	39 (76)
Interrupted	0 (0)	1 (2)
Combined	4 (19)	11 (22)
Unknown	1 (5)	0 (0)
Suture material, *n (%)*		

Variable	**Cases with incisional hernia (*n* = 21)**	**Controls withou hernia (*n* = 51)**
Maxon	11 (52)	22 (43)
PDS	4 (19)	11 (22)
Monoplus	0 (0)	1 (2)
unknown	6 (29)	17 (33)
Medication, *n (%)*		
ACE-Inhibitors	1 (2)	7 (14)
Steroids	0 (0)	2 (4)
NSAR	3 (6)	8 (16)
Co-morbidities, *n (%)*		
Diabetes	3 (14)	3 (6)
Chronic lung disease	2 (10)	0 (0)
Aneurysm	1 (5)	0 (0)
Collagen diseases	0 (0)	0 (0)
Previous hernia (inguinal or umbilical)	4 (19)	5 (10)
Chronic renal disease	0 (0)	1 (2)
Anemia	0 (0)	5 (10)
Albumin pre-operative (g/l), *mean ± SD*	44.2 ± 2.9	45.2 ± 2.9
Time between surgery and incisional hernia development, *n (%)*		
<6 months	8 (38)	-
6–12 months	5 (24)	-
>12 months	7 (33)	-
unknown	1 (5)	-
Follow-up (months), *mean ± SD*	22.0 ± 6.4	26.2 ± 5.7

n: absolute number; SD: standard deviation

Table 2

Top ranked antibodies with p-values <0.05 and log2 ratios of > 1 or < −1. Log_2 represents a 2-fold change.

Antibody name	Gene name	log_2 ratio	p-value
RNA polymerase III transcription initiation factor 50 kDa subunit	BRF2	1.78	0.001
Calreticulin 3	CALR3	1.36	0.017
Estrogen receptor 1	ESR1	1.32	0.021
Harvey rat sarcoma viral oncogene homolog	HRAS	1.20	0.003
Fibronectin 1	FN1	1.18	0.021
Heparan sulfate proteoglycan 2	HSPG2	1.17	0.001
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	1.13	0.042
Cyclin-dependent kinase inhibitor 1B	CDKN1B	1.12	0.017
Filamin A, alpha	FLNA	1.07	0.019
Selectin E	SELE	1.04	0.030
TBC1 domain family, member 3	TBC1D3	1.01	0.041
Ras-related protein Rap-1A	RAP1A	1.01	0.036
Interleukin 12A	IL12A	1.00	0.006
Post-GPI Attachment To Proteins 3	PERLD1	−1.05	0.038
Conserved helix-loop-helix ubiquitous kinase	CHUK	−1.05	0.010
Coagulation factor II (thrombin)	F2	−1.05	0.022
Protocadherin-15	PCDH15	−1.05	0.018
P-cadherin	CDH3	−1.07	0.001
Colon cancer secreted protein-1	CCSP-1	−1.12	0.016
Epithelial cell adhesion molecule	EPCAM	−1.13	0.010
SRA stem-loop-interacting RNA-binding protein	SLIRP	−1.22	0.022
Chemokine (C-C motif) ligand 21	CCL21	−1.30	0.019
Defensin alpha 1	DEFA1	−1.34	0.033
Sex hormone-binding globulin	SHBG	−1.49	0.002
Proteasome subunit beta type-5	PSMB5	−1.52	0.011

Table 3

Antibodies with p-values <0.05 and log2 ratios between +/−0.5 and +/−1. Log_2 represents a 2-fold change.

Kallikrein Related Peptidase 5	KLK5	0.98	0.042
Phospholipid scramblase	PLSCR1	0.93	0.017
Checkpoint Kinase 1	CHEK1	0.92	0.01
Thrombospondin 3	THBS3	0.92	0.034
Aryl Hydrocarbon Receptor Nuclear Translocator	arnT	0.92	0.037
YES Proto-Oncogene 1, Src Family Tyrosine Kinase	YES1	0.9	0.032
Tumor Protein, Translationally-Controlled 1	TPT1	0.84	0.005
Lactalbumin Alpha	LALBA	0.82	0.02
Collagen Type I Alpha 1	COL1A1	0.82	0.03
Von Hippel-Lindau Tumor Suppressor	VHL	0.78	0.01
SMAD Family Member 2	SMAD2	0.78	0.004
Interleukin 1 Beta	IL1B	0.78	0.039
Cytochrome P450 Family 2 Subfamily C Member 8	CYP2C8	0.77	0.036
Fibrillin 2	FBN2	0.77	0.022
Enolase 1	ENO1	0.77	0.039
PH Domain And Leucine Rich Repeat Protein Phosphatase	PHLPPL	0.73	0.037
Deoxycytidine Kinase	DCK	0.71	0.048
Cut Like Homeobox 1	CUX1	0.69	0.039
Wingless-Type MMTV Integration Site Family, Member 7A	WNT7A	0.68	0.042
Membrane Spanning 4-Domains A7	MS4A7	0.66	0.035
Acyloxyacyl Hydrolase	AOAH	0.66	0.026
Oncostatin M	OSM	0.65	0.045
CTD Phosphatase Subunit 1	CTDP1	0.64	0.037
Forkhead Box A1	foxa1	0.63	0.045
MDM2 Proto-Oncogene	MDM2	0.61	0.043
G Protein Subunit Alpha I3	GNAI3	0.59	0.015
Paraoxonase 1	PON1	0.58	0.04
Trefoil Factor 3	TFF3	0.54	0.035
GCSF, Colony Stimulating Factor 3 (Granulocyte)	CSF3	0.53	0.039
Myotubularin Related Protein 11	MTMR11	0.52	0.031
Granulin	GRN	−0.51	0.048
C-C Motif Chemokine Ligand 20	CCL20	−0.55	0.031
Glucokinase	GCK	−0.58	0.046
Stromal Cell Derived Factor 4	SDF4	−0.59	0.024
Cyclin B1	CCNB1	−0.59	0.049
Phosphoglycerate Mutase 1	PGAM1	−0.61	0.028
NCK Adaptor Protein 1	NCK1	−0.62	0.007

Neuropeptide Y	NPY	−0.62	0.024
Prostaglandin-Endoperoxide Synthase 1	PTGS1	−0.62	0.03
Nidogen 1	NID1	−0.63	0.04
Hypoxia Inducible Factor 1 Alpha Subunit	HIF1A	−0.66	0.039
Deleted In Malignant Brain Tumors 1	DMBT1	−0.66	0.031
Mitogen-Activated Protein Kinase-Activated Protein Kinase 3	MAPKAPK3	−0.67	0.04
Ribosomal Protein	RPS	−0.69	0.024
Epidermal Growth Factor Receptor	EGFR	−0.69	0.05
Minichromosome Maintenance 8 Homologous RecombinationRepair Factor	MCM8UV	−0.71	0.041
Parkinsonism Associated Deglycase	PARK7	−0.71	0.045
Protein Phosphatase 2 Regulatory Subunit B, Alpha	PPP2R2AUV	−0.72	0.036
Prohibitin	PHB_SDI	−0.77	0.028
Peptidylprolyl Isomerase Like 3	PPIL3UV	−0.77	0.035
Homeobox D13	HOXD13	−0.78	0.011
C-X-C Motif Chemokine Ligand 12	CXCL12	−0.81	0.031
Early Endosome Antigen 1	EEA1	−0.81	0.023
Orosomucoid 1	ORM1	−0.82	0.031
Transforming Growth Factor Alpha	TGFA	−0.82	0.04
Myocyte Enhancer Factor 2C	MEF2C	−0.88	0.034
Keratin 23	KRT23	−0.89	0.002
Collagen Type XXIV Alpha 1	COL24A1	−0.9	0.022
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	−0.92	0.026
Achaete-Scute Family BHLH Transcription Factor 1	ASCL1	−0.92	0.042
T-Cell Leukemia Homeobox 2	TLX2	−0.94	0.04

Table 4

KEGG sets with p-values <0.05.

	Number of genes in set	Number of unique genes observed	AUC	p-value
KEGG sets				
ECM receptor interaction	84	4	0.60	0.0002
Intestinal immune network for IgA production	48	1	0.41	0.0024
Progesterone mediated oocyte maturation	86	3	0.60	0.0062
Melanoma	71	4	0.58	0.0073
Bladder cancer	42	3	0.58	0.0084
Complement and coagulation cascades	69	1	0.39	0.0112
Ubiquitin mediated proteolysis	138	2	0.62	0.0175
Focal adhesion	201	8	0.55	0.0224
Regulation of actin cytoskeleton	216	4	0.56	0.0267
Pathways in cancer	328	12	0.54	0.0272
Toll like receptor signaling pathway	102	3	0.75	0.0005