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Altered mRNA Expression Due to Rectal Perforation in a Porcine Model – A Pilot Study

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Abstract. *Background: Anastomotic leakage is the most serious and unwelcome complication in rectal surgery. It has a great impact on postoperative morbidity and mortality. In this pilot study, changes of mRNA expression in blood were analyzed in an animal model designed to imitate anastomotic leakage. Materials and Methods: Twelve pigs were randomized into two groups: A sham-operated control group and an experimental group in which iatrogenic rectal perforation was performed. The changes in the mRNA expression at 4 hours after creating the perforation were studied. Microarray analysis was performed using Gene Chip whole porcine genome array. mRNA expression of 19,124 genes was investigated. Results: Significantly increased levels of genes with a fold change greater than 2 were found, including 276 coding for unknown proteins and 48 coding for known proteins. Eleven of those which coded for known proteins were up-regulated with a fold change >4. Conclusion: Eleven known genes were highly up-regulated after rectal perforation. These genes were mainly involved in inflammatory response, intracellular signaling and cell membrane regulation. Their corresponding proteins might potentially be clinical biomarkers of anastomotic leakage and should be evaluated in further clinical studies.*

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Key Words: Colorectal surgery, anastomotic leakage, gene expression analysis, microarray.

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Anastomotic leakage (AL) is a common and feared complication in colorectal surgery. The diagnosis of AL is often made late in the process due to the fact that there are no conclusive laboratory tests or X-ray examinations to determine such complication early in the process. C-Reactive protein is the most commonly used marker in laboratory tests, but it is clinically reliable only after 4-5 postoperative days. Computed tomography with anal enema is often unable to detect a leak in the early postoperative stage (1-4). Intraperitoneal microdialysis has been used in studies and has shown promising results but has not been used in clinical care for early diagnosis of AL (5-11). Cytokines are sensitive biomarkers for inflammation but not specific for the detection of AL (12-17).

mRNA analysis offers a new pathway in the search for laboratory markers specific for detecting AL. mRNA links genetic information from DNA to the ribosome in order to facilitate gene expression. RNA polymerase transcribes primary transcript mRNA into mature mRNA. Mature mRNA is then translated into a protein in the ribosome. mRNA genetic information is a sequence of nucleotides which are arranged into codons each consisting of three base pairs. Each codon encodes for a specific amino acid, except the stop codons, which terminate protein synthesis (18). Two other types of RNA contribute to protein synthesis, transfer RNA contributes in the recognition of the codon and ribosomal RNA plays a central role in the construction of protein chains (19). There are now commercially available microarrays for both human and animal specimens that can detect the expression of up to 20-30,000 genes through mRNA analysis (20-25).

In this pilot study, alterations of mRNA expression in blood samples due to rectal perforation were compared between an experimental group and a control group. The aim of this study was to identify a biochemical marker for the early detection of AL by analysing the changes in gene expression in whole blood in response to rectal perforation using a whole genome porcine microarray by an inductive strategy.

Materials and Methods

Animals. In this study, 12 healthy 3-month-old Swedish landrace pigs of both sexes were used, with mean body weight 28 kg (21-37). The pigs were housed at room temperature at a farm, with free access to standard porcine fodder before the experiment. They were kept under a 16-hour day and 8-hour night cycle. The experiment was approved by the Regional Animal Ethics Committee in Linköping (Dnr 174-3). The study was conducted in accordance with the guidelines of the European Union for the protection of animals used for scientific purposes. The animal experimentation in this study is reported according to the ARRIVE guidelines (26).

Anesthesia, fluid administration, ventilation and euthanasia. As pre-medication at the farm, the pigs were given azaperone [200 mg intramuscularly (*i.m.*); Elanco, Herlev, Denmark]. On arriving at the laboratory, anesthesia was induced by tiletamine (6 mg kg^{-1} *i.m.*; Virbac, Kolding, Denmark), zolazepam (6 mg kg^{-1} *i.m.*; Virbac) and azaperone (4 mg kg^{-1} *i.m.*). In addition, atropine (1.5 mg, *i.m.*; Mylan, Stockholm, Sweden) was given to prevent excessive salivation. The animals received two peripheral catheters (1.1 mm, Venflon Pro Safety; BD, Helsingborg, Sweden) in auricular veins. Propofol [1-2 mg kg^{-1} intravenously (*i.v.*); Fresenius Kabi, Uppsala, Sweden] was given if needed. The animals were orally intubated in the prone position with a 6-mm endotracheal tube (Covidien, Tullamore, Ireland). Anesthesia was maintained by propofol (10 mg kg^{-1} h^{-1} *i.v.*) and Petidin (1 ml kg^{-1} h^{-1}) applied by motorized syringe pumps (Alaris CC; Cardinal Health, Rulle, Switzerland). The depth of anesthesia was intermittently monitored by pain response. No muscle relaxants were given. Ringer's acetate (10 ml kg^{-1} h^{-1}, *i.v.*; Fresenius Kabi) and 10% glucose with 40 mM sodium and 20 mM potassium (0.5 ml kg^{-1} h^{-1}, *i.v.*; Fresenius Kabi) were administered by volume pumps (Alaris GP, CareFusion Corporation, Switzerland) to substitute for fluid loss. The pigs were ventilated using volume-control ventilation mode (PV 501, Breas Medical AB, Sweden) to achieve an arterial pCO_2 of 5.0-5.3 kPa and FiO_2 was adjusted to maintain arterial pO_2 at 12-18 kPa. The animals were placed on a thermal mattress and covered with a forced-air warming blanket. At the end of the experiment, euthanasia was performed with a rapid *i.v.* injection of 40 mmol potassium chloride (B. Braun, Danderyd, Sweden), and asystole and circulatory arrest were confirmed with ECG and blood pressure recordings.

Surgical preparation and measurements. A 4-Fr introducer was placed in the right carotid artery by open exploration for the measurement of systemic blood pressure as well as blood sampling. A midline abdominal incision was performed. A 14-Fr Foley catheter was inserted into the urinary bladder and fixed with a purse-string suture. Rectal perforation was performed with scissors. The perforation was located 8 cm above the anal verge. The diameter of the hole was 3 cm. The midline incision was sutured at the end of the procedure with a continuous suture.

Protocol. Using a sealed envelope system, the animals were randomized before the operation into two groups, with six in each group: An experimental group with rectal perforation; and a sham-operated control group. After the operation, there was an intervention-free period of 1 hour. Blood samples for mRNA analysis were taken before the laparotomy, and 4 hours after performing rectal perforation in PAXgene Blood RNA tubes (PreAnalytiX QIAGEN/BD, Hombrechtikon, Switzerland).

mRNA analysis. mRNA analysis was performed by the Bioinformatics and Expression Analysis, a core facility, which is supported by the Faculty Board of research at the Karolinska Institute and the Committee for Research at the Karolinska University Hospital, Stockholm, Sweden. RNA quality was assessed by RNA Integrity Number (RIN) and an RIN ≥9 was considered satisfactory for inclusion. RNA quantity of at least 3.5 μg was required for proceeding with expression analysis. Total RNA was isolated from PAXgene Blood RNA tubes with PAXgene Blood RNA Kit standard protocol on a QIAcube, Qiagen. Total RNA quality was assessed by Agilent Technologies 2200 Tapestation (Agilent Technologies, Waldbronn, Germany) and concentrations were measured by NanoDrop ND-1000 Spectrophotometer NanoDrop Technologies, Wilmington, DE, USA).

Labelling protocol: 150 ng of total RNA was used to generate amplified sense-strand DNA targets using Affymetrix WT Plus Kit (Affymetrix Inc., Santa Clara, CA, USA) followed by fragmentation and biotinylation.

Hybridization protocol: 2.2 μg of single-stranded DNA target was hybridized to Porcine Gene 1.0 ST Arrays in Affymetrix Gene Chip Hybridization Oven 645 (Affymetrix Inc.) (27). Hybridization, washing, and staining was carried out on Affymetrix GeneChip® Fluidics Station 450 (Affymetrix Inc.), according to the manufacturer's protocol. The fluorescent intensities were determined with Affymetrix GeneChip Scanner 3000 7G (Affymetrix Inc.). Protein identification was performed according to gene databases (www.uniprot.org)

Statistical analysis. Statistical analysis was performed with unpaired *t*-test with the assumption that the values were normally distributed, in R-interface software system, version 3.6.1. *p*-Values were adjusted for multiple testing using with Benjamini-Hochberg procedure. Values of $p<0.05$ were considered significant.

Results

Preoperatively and throughout the experiment, both groups had stable pulse, blood pressure and urine production. In total, 19,124 mRNA genes were investigated. A significant number of genes were found to be up-regulated and with a fold-change (FC)>2, including 276 genes which coded for unknown proteins and 48 genes which coded for known proteins. The majority of the annotated genes are involved in the inflammatory response, regulation of the membrane and intracellular signaling (Table I). Furthermore, 11 up-regulated genes were identified with FC>4 and these are presented separately by Probe Set ID in the text below and in Figure 1.

Table I. *Gene expression data for group comparison. Includes the Probe Set Identification Number (ID), fold change (FC) at 4 hours after surgery, p-value and protein description of the 48 mRNAs which coded for known proteins.*

Probe Set ID	FC	*p*-Value	Protein
15242239	11.701	0.000737	Tumor necrosis factor, alpha-induced protein 6
15218761	9.7341	0.0000275	Potassium inwardly-rectifying channel, subfamily J, member 15
15295924	6.6565	0.000212	Haptoglobin
15254582	6.3697	0.019335	Transcobalamin I (vitamin B12 binding protein, R binder family)
15287911	6.2881	0.000212	Activin A receptor, type IB
15254535	5.9668	0.001361	Membrane-spanning 4-domains, subfamily A, member 7
15330079	5.7942	0.0000794	Matrix metallopeptidase 1 (interstitial collagenase)
15285579	4.8529	0.000257	S100 calcium binding protein A8
15190807	4.7245	0.000212	Lipocalin 2
15280666	4.645	0.000342	S100 calcium-binding protein A9
15263686	4.2737	0.001065	Resistin
15249538	3.856	0.008008	Antileukoproteinase-like
15247299	3.4524	0.005042	Antileukoproteinase-like
15263641	3.3756	0.004251	EGF-like module containing, mucin-like, hormon receptor-like 1
15275209	3.2573	0.00047	AT-rich interactive domain-containing protein 5A-like
15322017	3.1694	0.0000751	Progestin and adipoQ receptor family member 3-like
15248234	3.1559	0.000212	Prion protein
15311674	3.0158	0.004586	Protein-glutamine gamma-glutamyltransferase K-like
15281489	2.9409	0.000212	BCL-2-like protein 15-like
15285575	2.9376	0.000231	S100 calcium binding protein A12
15247429	2.8959	0.003717	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)
15305270	2.795	0.027735	Sphingomyelin phosphodiesterase, acid-like 3B
15248514	2.7089	0.014077	Thrombomodulin
15270286	2.6795	0.007568	Interleukin 1 receptor, type II
15312575	2.5983	0.001713	Arginase, type II
15330095	2.4856	0.013268	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
15283223	2.4671	0.0000411	ATPase, H+ transporting, lysosomal 42 kDa, V1 subunit C1
15328685	2.4087	0.005984	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2-like
15328709	2.3975	0.011844	C4b-binding protein alpha chain-like
15303201	2.2921	0.005042	B-Cell CLL/lymphoma 3
15286684	2.2727	0.000212	Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase
15252920	2.2718	0.019833	Probable G-protein coupled receptor 141-like
15332619	2.2582	0.000713	Feline leukemia virus subgroup C cellular receptor 1
15297967	2.2421	0.003271	T-Cell-interacting, activating receptor on myeloid cells protein 1-like
15280703	2.1977	0.000212	S100 calcium binding protein A11
15322702	2.1955	0.004586	TEC protein tyrosine kinase
15190313	2.1901	0.024523	Toll-like receptor 4
15223879	2.1739	0.0000675	Scavenger receptor class B, member 1
15205901	2.1517	0.005746	M-Phase phosphoprotein 8
15320209	2.1511	0.007568	RNA-binding protein 47-like
15187496	2.12	0.0000235	Thioredoxin-related transmembrane protein 3
15253738	2.1063	0.002362	Glutathione S-transferase P 1-like
15277372	2.0858	0.003524	Inhibitor of DNA-binding 2, dominant negative helix-loop-helix protein
15230062	2.0697	0.000753	Scavenger receptor class B, member 1
15296514	2.0606	0.014064	Free fatty acid receptor 2
15282065	2.0536	0.001214	Rho GTPase-activating protein 29-like
15297331	2.0208	0.003717	Peptidoglycan recognition protein 1

Discussion

The porcine model for AL was a feasible model for the whole genome expression array study of whole blood using the Affymetrix porcine array. The mRNA in whole blood is derived mostly from lymphocytes (T-cells, B-cells, natural killer cells and monocytes). Circulating blood cells may carry valuable information in their RNA expression profile that may be indicative of incipient inflammatory processes (19). The Affymetrix microarray method was chosen for assessing mRNA expression in this study. In doing so, financial expenditure was taken into account and

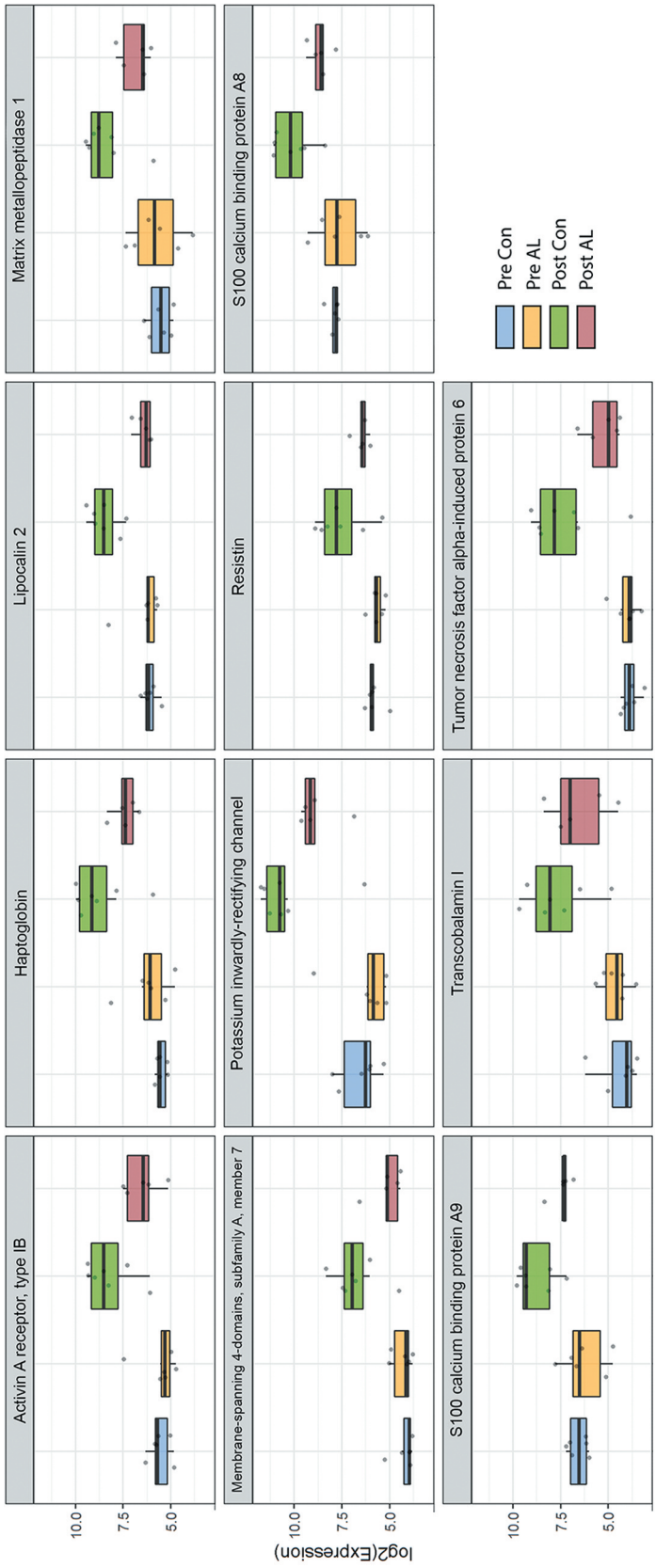

Figure 1. *Boxplot showing the proteins corresponding to the 11 known genes with the highest fold changes (>4) in mRNA expression at 4 hours after surgery in the sham-operated control (CON) animals and those with anastomotic leakage (AL). Preoperative and postoperative expression levels are shown. The black line in the boxes indicates the median value. The boundaries indicate the highest and lowest expression. For a better overview, fold-change values were log2-transformed. All statistical calculations were performed on non-logarithmic values.*

consideration was given to the possibility of obtaining a large number of mRNA assays, while at the same time being aware of the advantages of other commercially available methods (27). To measure how much mRNA signal changes from an initial value (before rectal perforation) to a final value (4 hours after rectal perforation) unlogged FC was used. Among the 1,9124 mRNA genes which significantly changed 4 hours after the perforation, 276 coded for unknown proteins and 48 known proteins. The 11 up-regulated genes with a high FC (over 4) out of the genes that coded for a known protein are known to be involved in increased inflammatory and immunological response, intracellular signaling and cell membrane regulation. All these findings are consistent with the pathophysiological process of acute inflammation/infection that occurs immediately after rectal perforation. mRNA transcript with ID 15242239 and corresponding protein tumor necrosis factor-alpha induced protein 6, was the protein with the highest FC, and is a secretory protein member of the hyaluronan-binding protein family. The hyaluronan-binding domain is known to be involved in extracellular matrix stability and cell migration. This gene can be induced by pro inflammatory cytokines such as TNF-α and interleukin-1 (28-30). TNF-α and interleukin-1 are proteins that rise rapidly in the blood after AL (3, 10, 15).

The protein encoded by the gene with ID 15218761 is an integral membrane protein and inward-rectifier type potassium channel. The encoded protein regulates the potassium flow into the cell, as well as sodium potassium homeostasis but its function in the inflammatory process occurring after AL is still unknown (31). The ID of the gene with the third-highest FC in expression corresponds to the protein haptoglobin. This protein binds free plasma hemoglobin, allowing degradative enzymes to gain access to hemoglobin, preventing the loss of iron through the kidneys, thus protecting the kidneys from damage by hemoglobin. The role of haptoglobin in the inflammatory process is to attract a monocyte to the site of inflammation (28-30, 32). The main group of enzymes responsible for collagen and protein degradation in the extracellular matrix is the matrix metalloproteinases (MMPs). MMP-1 was significantly up-regulated in the group with AL, consistent with previous studies (33). MMP-9 is also associated with AL and can be increased by lipocalin-2 when AL occurs (34, 35). Resistin is a protein which is up-regulated in inflammatory processes and even in carcinogenesis, like S100A and B and activin receptor type 1B (36-38).

Blood samples were analyzed 4 hours after a rectal perforation in pigs with the intention of emulating the reaction in the body immediately after an AL. The study concentrates on the 48 mRNAs encoding for a known protein and attempts to understand the initial pathophysiological processes immediately after the leakage. Ultimately, it would be beneficial to identify markers with high clinical sensitivity and specificity even during the first 3 days after an AL in order to treat it. Based on the results of this study, further studies should be conducted with an aim of finding appropriate clinical laboratory markers for AL.

Limitations of the study. The porcine and human gastrointestinal tracts are very similar and, therefore, pigs were used in this study; however, specific proteins may vary significantly between species. The majority of mRNAs found to be up-regulated in this study coded for unknown proteins. Their significance is still to be explored, as their function is still unknown. The study was only performed during the first 4 postoperative hours. AL usually develops gradually due to local ischemia in the first 3-5 postoperative days.

Conclusion

mRNA expression of 11 known genes was up-regulated in pigs after surgical creation of a rectal perforation. These genes were mainly involved in inflammatory response, intracellular signaling and cell membrane regulation. Their corresponding proteins might potentially be clinical biomarkers of AL and should be evaluated in further studies.

Conflicts of Interest

None of the Authors have any relevant conflicts of interest to declare.

Authors' Contributions

Ioannis Oikonomakis, David Brodin and Kjell Jannson designed the study. David Brodin performed the statistical analysis. Ioannis Oikonomakis, Tal Hörer, Kristofer Nilsson, Jenny Seilitz and Kjell Jansson performed the study. All the Authors analyzed the results and contributed to writing the article.

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