Postoperative Rise of Circulating Mitochondrial DNA Is Associated with Inflammatory Response in Patients following Pancreaticoduodenectomy

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Keywords

Mitochondria · DAMPs · SIRS · Pancreatic surgery

Abstract

Introduction: Accumulation of plasma mitochondrial DNA (mtDNA) following severe trauma has been shown to correlate with the development of systemic inflammatory response syndrome (SIRS) and may predict mortality. Our objective was to investigate the relationship between levels of circulatory mtDNA following pancreaticoduodenectomy (PD) and the postoperative course. **Methods:** Levels of plasma mtDNA were assessed by real-time PCR of the mitochondrial genes *ND1* and *COX3* in 23 consecutive patients who underwent PD 1 day prior to surgery, within 8 h after surgery, and on postoperative day (POD)1 and POD5. The abundance of mtDNA was assessed relative to preoperative levels and in relation to parameters reflecting the postoperative clinical course. **Results:** When pooled for all patients, the circulating mtDNA levels were significantly increased after surgery. However, while a significant (at least >2-fold and up to >20-fold) rise was noted in 11 patients, no change in mtDNA levels was noted in the other 12 following surgery. Postoperative rise in circulating mtDNA was associated with an in-

creased rate of postoperative fever until day 5, decreased hemoglobin and albumin levels, and increased white blood cell counts. These patients also suffered from increased rates of delayed gastric emptying. No significant differences were demonstrated in other postoperative parameters. **Conclusion:** Circulating mtDNA surge is associated with an inflammatory response following PD and may potentially be used as an early marker for postoperative course. Studies of larger patient cohorts are warranted.

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Introduction

Abdominal surgery triggers an inflammatory response, the magnitude of which usually correlates with the extent of the surgical trauma [1–3]. Following major surgery, an initial hyperinflammatory response may evolve into systemic inflammatory response syndrome (SIRS), and occasionally lead to multiple organ dysfunction syndrome (MODS) [4, 5]. It was previously shown that early development of SIRS after surgery is associated with an adverse postoperative clinical course, a higher rate of severe morbidity, and early postoperative mortal-

ity [6–9]. Detection of patients at risk of postoperative SIRS may allow for prompt intervention and potentially improves outcome [9, 10]. Unfortunately, early markers of inflammation that can indicate an upcoming complication are not readily available.

Studies have shown that severe trauma induces the release of mitochondrial damage-associated molecular patterns (DAMPs), consisting of mitochondrial DNA and formyl peptides [11–14]. Mitochondrial DAMPs directly activate polymorphonuclear cells, leading to a sepsis-like state and neutrophil-mediated organ injury [11–14]. Moreover, following severe trauma, an increased level of circulating mitochondrial DNA (mtDNA) DAMPs has been associated with the development of acute respiratory distress syndrome (ARDS), SIRS, MODS, and mortality [14–17]. A postoperative surge of circulating mtDNA following major abdominal surgery such as pancreaticoduodenectomy (PD) which, by necessity, is associated with tissue trauma, may play a role in the development of postoperative SIRS and other consequent complications.

We assessed whether postoperative levels of circulating mtDNA are associated with the clinical course of patients who underwent PD for borderline resectable pancreatic adenocarcinoma (PDAC).

Materials and Methods

Patients

Included in this prospective cohort study are 23 patients that underwent PD for borderline resectable PDAC [18] between December 2014 and March 2016. Until recently, neoadjuvant treatment was not routine for borderline resectable PDAC and none of the patients received preoperative chemotherapy. Also, our policy was to avoid preoperative biliary interventions to reduce infectious and inflammatory complications, so no patient underwent preoperative biliary drainage. Therefore, no inflammatory events such as pancreatitis or cholangitis occurred prior to surgery. The mean preoperative serum amylase level was 89 ± 37 U/L and no patient had levels above the normal values. Demographics and preoperative data were collected from patients prior to surgery. In 2 patients, the operation was aborted due to metastatic spread found at examination; they were excluded from the study. Peri- and postoperative data, as well as circulating mtDNA levels, were collected prospectively.

Assessment of Circulating mtDNA Levels

Ten milliliters of peripheral blood were sampled 1 day prior to surgery and on postoperative day (POD)1 and POD5. The blood was originally placed in BD Vacutainer K2E (EDTA)-containing tubes (Becton, Dickinson & Co., Plymouth). Samples were centrifuged within 1 h of collection at an RCF of 1,000 for 10 min at room temperature, and 200 μ L of the plasma fraction was decanted and processed using the QIAamp DNA blood mini kit (QIAGEN,

Hilden, cat. No. 51106). Quantitative real-time polymerase chain reaction (qPCR) was performed with fast SYBR Green master mix (Applied Biosystems, Part No. 4385612) using a StepOnePlus real-time PCR system (Applied Biosystems, S/N 272008358), according to the manufacturer's protocol, to quantify selected ~200 bp sequences corresponding to the NADH dehydrogenase 1 (ND1) and cytochrome C oxidase subunit 3 (COX3) genomic regions. Primer sequences were as follows: ND1: F- ATACCCATGGCCAA-CCTCCT, R- GGGCCTTTGCGTAGTTGTAT; COX3: F- AG-GCATCACCCCGCTAAATC, R- CCGTAGATGCCGTCGG-AAAT. The abundance of mtDNA was expressed as a threshold cycle (T_c) and as relative to the level before surgery. Of note, a higher T_c represents a lower level of mtDNA DAMPs.

Clinical variables were recorded continuously as part of the standard postoperative monitoring and specifically at the time of blood drawn for mtDNA. Laboratory variables were obtained in parallel to blood sampling for mtDNA. Postoperative complications were evaluated and graded using the Clavien-Dindo (CD) classification of surgical complications [19]. Specific PD-related complications such as delayed gastric emptying, pancreatic fistula, postpancreatectomy hemorrhage (PPH), etc. were also assessed. SIRS was defined according to the American College of Chest Physicians (ACCP)/Society of Critical Care Medicine (SCCM) definition [20]. MODS was defined as a Denver postinjury multiple-organ failure score of ≥ 4 [21]. This scoring system rates the dysfunction of 4 organ systems (i.e., pulmonary, renal, hepatic, and cardiovascular) which are assessed daily during a patient's stay in the intensive care unit (ICU).

Statistical Analysis

Statistical analysis was performed using the IBM SPSS statistics data editor v25. Continuous data are expressed as median values with the corresponding SD. Student's t test was used for continuous data and the χ^2 test for categorical data. Patients were divided into those with and without a significant increase in mtDNA following surgery, a sample size of at least 10 patients in each group was sufficient for 0.8 power with at least 10% difference ($\alpha = 0.05$) in the blood indices (continuous parameters) examined. $p < 0.05$ was considered statistically significant.

Results

Between December 2014 and March 2016, 23 patients (7 females; mean age 67 years, range 46–83 years) underwent an elective pancreatic resection for borderline resectable PDAC by an open approach. Patient characteristics as well as operative and postoperative data are supplied in Table 1. The average operative time was 444 ± 161 min (range 312–798 min) and mean estimated blood loss was 245 ± 170 mL. Three patients received packed cells during surgery (1 received 2 units and 2 received 1 unit each). In 7 patients, superior mesenteric vein/portal vein resection and reconstruction were required to achieve complete tumor extirpation. All patients were admitted to the ICU following surgery. Average time in

Fig. 1. Overall pre and postoperative levels of circulating mtDNA. Abundance of plasma mtDNA as evaluated using the ND1 and COX3 amplicons and expressed as threshold cycle (Tc) preoperatively (Ref) and on postoperative days 0, 1, and 5 (D0, D1, and D5) for all patients. Note that higher Tc represents lower levels of mtDNA. Data represent mean $\pm$ SD of 2 biological repeats performed in triplicate. * $p < 0.05$.

Table 1. Patient demographics and operative characteristics

	All patients (<i>n</i> = 23)	Postop mtDNA increase (<i>n</i> = 11)	No mtDNA increase (<i>n</i> = 12)	<i>p</i> value
Age, years	67.4 $\pm$ 9.6	69.4 $\pm$ 8.2	65.5 $\pm$ 10.3	0.35
Males/females, <i>n</i> (ratio)	16/7 (2.28)	7/4 (1.75)	8/3 (2.6)	0.72
BMI	25.1 $\pm$ 4	25.8 $\pm$ 3.7	24.4 $\pm$ 4.2	0.44
Comorbidity				
HTN	12 (52.1)	7 (63.6)	5 (41.6)	0.29
DM	12 (52.1)	7 (63.6)	5 (41.6)	0.29
IHD	3 (13)	2 (18.1)	1 (8.3)	0.48
CKD	1 (4.3)	1 (9)	–	–
COPD	2 (8.6)	2 (18)	–	–
Prior abdominal surgery	7 (30.4)	2 (18)	5 (41.6)	0.22
Duration of surgery, min	444 $\pm$ 161	462 $\pm$ 148	428 $\pm$ 170	0.063
Pyloric preservation	11 (47.8)	4 (36.3)	7 (58.3)	0.29
Estimated blood loss, mL	252 $\pm$ 166	318 $\pm$ 203	191 $\pm$ 86	0.04
Blood transfusion	3 (13)	2 (18)	1 (8.3)	0.28
Vascular resection	10 (43.4)	5 (45.4)	5 (41.6)	0.95
Extubated at the end of surgery	15 (65)	6 (54)	9 (75)	0.35

Values are expressed as *n* (%) or mean $\pm$ SD, unless otherwise indicated. BMI, body mass index; HTN, hypertension; DM, diabetes mellitus; IHD, ischemic heart disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease.

Fig. 2. Grouped differences in pre- and postoperative levels of circulating mtDNA. Relative abundance of plasma mtDNA as evaluated using the ND1 and COX3 amplicons. Data represent relative abundance of mtDNA on postoperative days 0, 1, and 5 (D0, D1, and D5) in relation to the preoperative levels in patients with >2-fold increase ($n = 11$; left) and those with no increase ($n = 12$; right).

ICU was 2.6 days (range 1–17 days). Nine patients had SIRS during the first 5 days following surgery, but no patient had MODS. Six patients (26%) suffered from major postoperative complications ($CD \geq 3$), while 17 had either minor ($CD < 3$) complications or an uneventful course. Two patients (8.6%) underwent reoperation during the immediate postoperative course. Mean length of hospital stay was 12 ± 3 days (range 8–21 days). One patient was rehospitalized within 30 days of surgery. There was no 30-day mortality. In all patients, an R0 resection was achieved.

Peripheral blood was drawn prior to surgery, within 8 h from operation, on POD1 and POD5. For each patient, mtDNA levels, depicted by the mitochondrial genes *ND1* and *COX3*, were quantified at the indicated time points as PCR threshold cycle (T_c), with each cycle doubling the DNA quantity until a detection threshold is achieved. mtDNA levels were also assessed as fold-change in relation to preoperative levels (Fig. 1). Overall, the mean mtDNA levels were significantly increased 8 h after surgery, on POD1 and POD5, compared to the preoperative levels (*ND1*: POD0, POD1, and POD5 fold-changes of 2.38 ± 7.85 , 2.85 ± 7.85 , and 2.44 ± 9.12 ; *COX3*: POD0,

POD1, and POD5 fold-changes of 4.03 ± 11.6 , 3.73 ± 7.1 , and 3.9 ± 8.8 , respectively; Fig. 1).

In 11 patients, the levels of circulating mitochondrial DNA increased significantly already on POD1 (at least >2-fold) compared to control levels (Fig. 2), while in the other 12 patients, no increase was demonstrated.

No significant differences in age, gender, body mass index (BMI), and comorbidities were noticed between the groups (Table 1). Patients with a postoperative rise in circulating mtDNA bled more during surgery but did not require excess amount of blood units. Other operative parameters including procedure length, the need for vascular resection, pyloric preservation, and ability to undergo extubation at the end of surgery were all comparable between the groups.

Postoperative increases in circulating mtDNA were associated with higher rates of postoperative fever, up until POD5, decreased hemoglobin levels, increased white blood counts, and decreased albumin levels (Table 2). These patients also suffered from increased rates of delayed gastric emptying. The rate and severity of other postoperative complications including pulmonary complications, surgical-site infection, pancreatic fistula, PPH,

Table 2. Postoperative course

	All patients (<i>n</i> = 23)	mtDNA increase (<i>n</i> = 11)	No mtDNA increase (<i>n</i> = 12)	<i>p</i> value
Fever until POD5	8 (34.7)	6 (54.5)	2 (16.6)	0.05
Hb level				
Preoperative, g/dL	11.97±1.97	12.26±1.43	11.87±1.03	0.39
POD1, g/dL	10.6±1.67	9.85±1.4	11.2±1.44	0.048
POD3, g/dL	10.27±1.6	9.3±1.62	10.83±1.66	0.047
POD5, g/dL	10.48±1.4	9.84±1.34	10.9±0.97	0.07
WBC count				
Preoperative, 10 ³ /μL	9.18±2.38	8.64±1.93	9.57±2.59	0.43
POD1, 10 ³ /μL	14.9±5.7	17.46±6.99	12.6±2.61	0.042
POD3, 10 ³ /μL	12.02±5.02	14.4±5.07	10.02±3.17	0.043
POD5, 10 ³ /μL	11.2±4.36	13.15±5.11	9.38±2.39	0.047
Albumin level				
Preoperative, g/L	36.4±3.9	36.17±5.87	37.3±2.21	0.57
POD1, g/L	26±3.9	24.3±4.05	27.4±3.1	0.046
POD3, g/L	25.2±3.5	23.7±2.96	26.6±3.4	0.041
POD5, g/L	26.18±3	25.1±2.6	27.08±3	0.13
Pulmonary complications	5 (21.7)	3 (27.2)	2 (16.6)	0.65
Delayed gastric emptying	9 (39.1)	7 (63.6)	1 (16.6)	0.02
Pancreatic fistula	4 (17.3)	2 (18)	2 (16.6)	0.92
Postpancreatic hemorrhage	2 (8.7)	1 (9)	1 (8.3)	0.95
Leak of enteric/biliary anastomosis	1 (4.35)	0	1 (8.3)	–
Postoperative complications				
CD ≥3	6 (26)	2 (18)	4 (33.2)	0.4
CD <3	9 (39.1)	4 (36)	5 (41.6)	0.8
Surgical wound infection	4 (17.3)	1 (9)	3 (0.25)	0.31
Acute renal failure	1 (4.35)	1 (9)	0	–
Reoperation	2 (8.7)	1 (9)	1 (8.3)	0.95
Length of hospital stay, days	11.95±3	11.8±3.61	12.09±2.27	0.84

Values are expressed as *n* (%) or mean ± SD. Hb, hemoglobin; WBC, white blood cells; CD, Clavien-Dindo scale.

the need for reoperation, and others were comparable between the groups. No difference in the overall length of stay was found between the groups.

Discussion

Recent reports showed that elevation of circulating mtDNA DAMPs following severe trauma triggers an inflammatory response by activating circulating polymorphonuclear (PMN) cells via pattern recognition receptors (PRR) including toll-like receptor 9 (TLR9) [11, 12, 22], and is associated with adverse patient outcome [11, 14, 23]. Simmons et al. [14] established a striking correlation between an early mtDNA rise in severely injured patients and later SIRS, MODS, and even mortality. This was supported by other reports demonstrating that increased lev-

els of circulating mtDNA were associated with the development of respiratory distress syndrome in trauma and sepsis patients [17] as well as mortality of ICU patients [24]. This raised the possibility of using mtDNA as a biomarker for the early detection of patients at risk of developing a hyperstimulated inflammatory response and adverse outcomes.

The goal of this study was to assess whether an elective surgery, which consists of significant but controlled tissue trauma, induces a surge of circulating mtDNA that correlates with SIRS and postoperative clinical course. Aiming to provide a preliminary proof of concept, we sought patients who underwent a reproducible major surgery that included extensive tissue dissection and organ resection. Open PD is a high-magnitude surgery, among the most complicated in general surgery, requires a relatively large abdominal incision, a wide tissue dissection, and re-

section of organs including the pancreas, duodenum, and bile duct. Moreover, this surgery is highly structured, with little variation between cases. This makes PD ideal for studying variations between patients in response to trauma. We specifically chose borderline resectable cases. These usually require an even wider tissue dissection, especially in cases in which vascular resection is needed. Even though the tissue trauma in elective surgery occurs in a controlled setting, we believe that the release, effect, and sequestration of DAMPs in such settings may differ between patients and affect the clinical course. Evaluating the mtDNA plasma levels preoperatively, 8 h after surgery, and on POD1 and POD5 corresponded with the study designs of Simmons et al. [14] and McIlroy et al. [25].

When pooled for all patients, the circulating mtDNA levels increased >2-fold following surgery, starting within 8 h after surgery using both markers (the mitochondrial genes *ND1* and *COX3* [14]). In all patients in whom mtDNA levels increased on POD0, these levels were maintained at POD5. When examined specifically, a clear dichotomy was noted: 12 had no significant rise in mtDNA at any time point examined but the other 11 showed a significant increase (in some, >20-fold). In their original report, Zhang et al. [11] demonstrated that following severe uncontrolled trauma, plasma mtDNA levels were elevated >1,000-fold compared to in healthy individuals. Later reports demonstrated a milder increase of 10- to 30-fold [13, 25], similar to the postoperative rise in some of our patients. Since no differences in demographics, medical history, or any significant surgery-related factors were demonstrated between the groups, it is probable that this rise in mtDNA is related to inherent factors that affect either the release or scavenging of mtDNA. McIlroy et al. [25] demonstrated that, in trauma surgery, the postoperative levels of mtDNA were not associated with markers of tissue necrosis but with surgical invasiveness.

Here, since all patients underwent the same surgery, other yet-to-be-investigated factors must have influenced the sustained mtDNA rise in some of the patients. Since the aim of this study was to evaluate whether an increase in the levels of circulating mtDNA as a response to controlled tissue trauma may relate to the extent of the inflammatory response, we were mainly interested in evaluating the difference between pre- and postoperative levels. Assessing the basal levels of mtDNA in relation to healthy individuals or to various other factors, including demographics, comorbidities, or disease-related factors, was beyond the scope of this study. Nevertheless, in our

cohort, the abundance of mtDNA prior to surgery was diverse, as demonstrated by the broad range of PCR cycle of detection (range 17.4–23.9 in *ND1* and 15.7–24.7 in *COX3*). This pattern was also demonstrated in other studies that assessed levels of mtDNA in the plasma of different patient populations; in all, the variance was high [14, 26]. Nonetheless, we could not define any significant correlation between basal mtDNA levels and demographic or disease-related factors. Factors influencing the basal levels of circulating mtDNA warrant a separate study.

The postoperative rise in circulating mtDNA was associated with fever and elevated markers of inflammation including leukocytosis, anemia, and hypoalbuminemia as well as a higher rate of delayed gastric emptying. Delayed gastric emptying is a known complication following PD and is usually associated with other complications, such as an anastomotic leak, leading to increased inflammatory response and sepsis [27]. Although these parameters are of great importance and may significantly influence the postoperative course, we were not able to demonstrate differences in overall length of stay or other PD-related complications. We believe this may have been related to both the small cohort size and the fact that some of the PD-associated complications were not necessarily associated with the increased inflammatory response (mainly in patients with a well-drained surgical site). An anastomotic leak is a good example, as in many patients systemic findings were limited. Previous studies have already shown that early increases in other inflammatory markers such as TNF α , IL-6, and C-reactive protein may predict SIRS after an elective abdominal surgery [2]. According to data from mouse models [11, 12], we believe that mtDNA, a DAMP directly related to the extent of tissue injury, may not only be a marker for the development of SIRS but also may play a role in initiating it.

This study has the limitation of a small cohort size. However, the cohort was homogenous in terms of disease burden and extent of surgery. Our aim was to provide an initial proof of concept for the potential use of circulating mtDNA level as an early biomarker of a postoperative inflammatory response.

To conclude, elevation of circulating mtDNA following elective PD is associated with an increased inflammatory response. Further studies, with larger cohorts and other surgical procedures are warranted, to establish circulating mtDNA as an early biomarker of inflammation and postoperative complications in elective surgical procedures.

Statement of Ethics

The study was approved by The Tel-Aviv Sourasky Medical Center Institutional Review Board, approval No. 0740-14-TLV. All patients included in this study signed a written informed consent.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

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