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Circulating Microvesicles Mediate the Inflammatory Response and Coagulopathy in Coronary Artery Bypass Graft Surgery

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Circulating microvesicles; Coronary artery bypass grafting; Cardiopulmonary bypass; Inflammation; Coagulation; Myocardical dysfunction

[&]Abbreviations:

cMVs: Circulating Microvesicles; CABG: Coronary artery bypass grafting; CPB Cardiopulmonary bypass; BNP: Brain natriuretic peptide; CRP: C-reactive protein; Mb: Myoglobin; PS: Phosphatidylserine

1. Abstract

Coronary Artery Bypass Grafting (CABG) is inevitably associated with some level of activation of the coagulation and inflammation systems. The purpose of this research was to access the circulating Microvesicles (cMVs) possible association with inflammatory response, coagulopathy in patients with on- and off- pump Coronary Artery Bypass Graft Surgery (CABG). Firstly, microvesicles obtained from the patients' plasma before (A0/B0) and 24 hours after (A1/B1) CABG with or without the use of Cardiopulmonary Bypass (CPB) (n=10 on-pump (A), n=10 off-pump (B), before surgery (A0/B0), 24 hours after surgery (A1/B1)). The microvesicles were analyzed by Flow cytometry. iTRAQ (isobaric tags for relative and absolute quantitation) analysis was performed at the levels of proteins. In result, the level of cMVs showed parallel changes with the levels of Brain Natriuretic Peptide (BNP), C-reactive protein (CRP), and Myoglobin (Mb) in the groups. The microvesicles from the Endothelial (EMP), Platelets (PMP), white blood cells (LMP) were all detected by makers of CD144, CD41a, CD45, but CD41a PMP and CD144 EMP levels enhanced significantly after on- and off-pump CABG, especially in the on-pump group. Further, iTRAQ analysis showed that the changing proteins contained in the microvesicles were mostly associated with the responses of inflammation and coagulation. Further, GO function classification and the KEGG pathway also revealed the same results. In conclusion, the microvesicles played a key role in the processes of inflammation and coagulation in CABG patients.

2. Introduction

Cardiovascular disease remains the leading of death in the world. Coronary Artery Bypass Grafting (CABG) has became the commonly performed surgical procedures, and reduced mortality in patients with severe coronary artery disease [1-3]. Early, Coronary Artery Bypass Grafting (CABG) with the use of cardiopulmonary bypass ("on pump") can provide a controlled condition under cardioplegic arrest. However, increasing numbers of researches reported that many deleterious effects were observed in patients undergoing on-pump technique, such as "systemic inflammatory response, coagulopathy and end-organs dysfunction". In the past decades, Coronary Artery Bypass Grafting (CABG) without the use of cardiopulmonary bypass ("off pump") has been developed to reduce the postoperative complications. Recently, accumulating studies have demonstrated a reduction in additional complications and the mortality in patients undergoing off-pump compared to on-pump approach [4-10].

In clinical, we often see more inflammatory response, coagulopathy and myocardical dysfunction after Coronary Artery Bypass

Grafting (CABG) with/without the use of cardiopulmonary bypass. And these effects are responsible for most of the morbidity after surgery. But there are no accurate prediction makers to access the post-CABG clinical outcomes [1, 11].

Microvesicles are released from the plasma membrane of cells by outward budding of membrane vesicles, following disruption of the natural asymmetrical distribution of membrane phospholipids, under conditions of cell activation, mechanical stress, and apoptosis. They retain surface antigens specific to their parent cell, and contain protein, RNA, miRNA, and lipids derived from the parent cell. Now microvesicles are widely recognized as key players in cell-to-cell communication. Moreover, it is ascribed important role to the process of coagulation and inflammation [12-15]. Here, we focused on the role of microvesicles and discussed their emergent role in mediating activation and response to inflammation, coagulation and myocardical dysfunction during the Coronary Artery Bypass Grafting (CABG).

3. Materials and Methods

3.1. Patient Population and Plasma Collection

Twenty patients undergoing cardiac surgery, either conventional CABG with Cardiopulmonary bypass (CPB) (n=10) or CABG without CPB (n=10), at the cardiac center of Airforce General Hospital were included in this study. This clinical study was conducted in accordance with the Ethics Guidelines for Research Involving Human Subjects or Human Tissue from the Airforce General Hospital. All of the patients provided written informed consent. Exclusion criteria were as follows: Patients having unstable conditions (recent acute coronary syndrome with peak Creatine kinase-MB (CK-MB) > 2 times the upper limit of normal), acute heart failure or left ventricular ejection fraction < 45%, requiring immediate surgery, severe hepatic and renal dysfunction, additional valve replacement surgery, or any evidence of infection. All involved patients were taking aspirin and/or clopidogrel/Ticagrelor, which was discontinued at least one week before the surgery, and instead, low molecular weight heparin was continued until 24 hours before surgery. (Table 1) summarized the characteristics of the main study groups. Cardiopulmonary bypass duration was 85.3±7.8 min. Midline sternotomy was performed in all patients.

Table 1: Demographic Data of patients

	on-pump	off-pump
Age(y)	66.2±11.5	67.6±8.4
Gender(M/F)	1/9	6/4c
Ejection Fraction(LVEF%)	54.5±10.7	56.2±12.4
Creatinine (mg/dl)	48.6±9.6	51.2±8.9
Diabetes(%)	70	60
Weight(kg)	67.3±12.7	64.5±10.8

aData are expressed as mean ±SEM or as % of population.

EDTA tubes (1 mg/ml EDTA) were used to collect venous blood from each group of patients at least 12 h before the induction of anesthesia (before surgery, on-pump A0/off-pump B0), and at 24 hours after surgery (on-pump A1/off-pump B1). Samples were kept in a refrigerator at 4°C. To obtain platelet-free plasma samples, the samples were first centrifuged at 1, 600 ×g for 10 min and then at 16, 000 ×g for 30 min, which was followed by filtration through a 0.22-μm Super Membrane (Pall Life Sciences, New York, USA) to remove apoptotic bodies; after that, the supernatant was isolated and stored at –80°C to be used in this study.

3.2. Microvesicle Isolation and Identification

Circulating Microvesicles (cMVs) were harvested as per the instructions on the ExoQuick serum exosome precipitation solution. The surface makers were evaluated with the bead-based flow cytometry technique. Microvesicles were reacted with a monoclonal antibody that targeted PS, all platelets, epithelial cells, and leukocyte-derived microvesicles (CD41a, CD144, and CD45). A Linotype control mixture matching the HRP conjugated anti-rabbit antibody was used (Cell Signaling Technology, Boston, USA, cat #7074). All of the antibodies were purchased from BD Biosciences, USA (CD41a, CD144, Annexin-V and CD45, cat #340931, #562242, #556420, #340943). We collected the data of microvesicles of different origins.

3.3. Protein Preparation

Microvesicles were collected as per the method described above. The samples were solubilized in lysis buffer (8M urea, 30 mM HEPES, 1 mM PMSF, 2 mM EDTA, and 10 mM Dithiothreitol (DTT)) using 5 min of sonication (pulse-on 2 s, pulse-off 3 s, power 180W), followed by centrifugation at 20, 000 rpm for 30 min at 4°c. The protein concentration of the clear supernatant was quantified with 2-D Quant kit. All of the proteins were kept at −80°C for further analysis.

3.4. Protein Digestion and iTRAQ Labeling

iTRAQ (Isobaric Tags For Relative And Absolute Quantitation) analysis was performed at Beijing Proteome Research Center (Beijing, China). Total protein (100 μg) was isolated from each sample solution and then the protein was digested with Trypsin Gold (Promega, Madison, WI, USA) with the 30:1 ratio of protein: trypsin at 37°C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plexiTRAQ reagent (Applied Biosystems, Carlsbad, CA, USA).

3.5. Strong Cation Exchange (SCX) Fractionation

Each fraction was resuspended in buffer A (25 mM NaH2PO4in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm Ul tremex

SCX column containing 5 μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 ml/min using buffer A for10min, 5–60% buffer B (25 mM NaH2PO4, 1 M KCl in 25% ACN, pH 2.7) for 27 min, and 60–100% buffer B for 1 min. The eluted peptides were desalted with a Strata X C18 column (Phenomenex).

3.6. MS Analysis by Q -Exactive

The eluted peptides were reconstituted with Mobile Phase A (2% acetonitrile, 0.1% FA). The mixture of peptides was loaded onto a C18 column and separated at a flow rate of 400 ml/min using a mobile phase B gradient of 5% for 10 min, 5%–30% for 30 min, 30%–60% for 5 min, and 60%–80% for 3 min; maintained at 80% for 7 min; returned to 5% for 3 min; and maintained at 5% for 7 min. The eluted peptides were detected using Q-Exactive, and MS data were acquired using a data-dependent top20 method by choosing the abundant precursor ions from the survey scan (350–20, 000 Da) using higher energy collision dissociation (HCD). Determination of the target value was based on automatic gain control (AGC). Survey scans were acquired at a resolution of 70,000 and the resolution for HCD spectra was set to 17,500. The iTRAQ analysis was performed as two technical replicates to gather reliable quantitative information.

GO is an international standardization system of gene function classification. It has 3 ontologies that can describe molecular function, cellular component, and biological process, respectively. In this study, functional annotations of the proteins were conducted using Blast2GO program against the non-redundant protein database (NR; NCBI).

KEGG PATHWAY is a database resource and a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks between the identified differentially expressed proteins in the on-pump and off-pump groups [16, 17].

Statistical analysis

All of the values are expressed as the mean± SEM. Statistical analysis was performed with student's two-tailed unpaired t test or one-way ANOVA using Graph Pad Prism 5. Statistical significance was accepted for all of the tests at $P < 0.05$.

4. Results

4.1. Clinical Characteristics

In the two study groups, there was a steady baseline and no significant differences in the clinical characteristics (Table 1), such as age, gender, weight, ejection fraction, and other demographic variables. In all of the patients in the on-pump group, the roller-type rotary pump was used. The time required for the surgery was similar in the two groups, and it was 6 h±45.8 min.

The levels of Brain Natriuretic Peptide (BNP), C-Reactive Protein (CRP) and Myoglobin (Mb), had no statistical differences in two groups before surgery. After surgery, these markers were obviously increased (Table 2), and compared with the off-pump group, the alterations were more significant in the on-pump CABG (Table 2).

4.2. Flow Cytometry for Different Microvesicles

Flow cytometry revealed that microvesicles stained by markers usually present on platelets (CD41a), endothelial cells (CD144), and white blood cells (CD45), and phosphatidylserine (PS) were already detectable in plasma samples at A0 (on-pump CABG before surgery) and B0 (off-pump CABG before surgery), and there were no statistical differences (Table.3, $P > 0.05$). However, the number of microvesicles stained by these markers tended to increase at A1 (on-pump CABG after surgery) and this tend was significant only for CD41a, CD144, phosphatidylserine (PS) (Table 4). Same results were also observed in the off-pump group at B0 (off-pump CABG before surgery) and B1 (off-pump CABG after surgery) (Table 5). All of the increased markers CD41a, CD144, phosphatidylserine (PS) were detected more frequently in the on-pump CABG group than in the off-pump CABG group (Table 6). These results suggested that the process of surgery could stimulate origin-related cells (endothelial cells, platelets, etc.), releasing more microvesicles. CABG was associated with a rapid increase in plasma microvesicles.

4.3. Classification of Differentially Expressed Proteins

Using the Proteome Discoverer software, we performed screening through mass spectrometry and a search via Mascot, and then, we performed quantitative analysis of the proteins with Uniprot-Human. Finally, we obtained a total of 201 regulated proteins from the on- and off-pump groups based on the databases. iTRAQ ratios >1.20 or <0.83 (P-value <0.05), respectively, were used to define proteins that were significantly up-regulated or down-regulated (Figure 1). In the on-pump CABG, 101 proteins showing changes were detected, and among them, 49 proteins were found to be up-regulated (Supplemental Table 1) and 52 proteins were found to be down-regulated (Supplemental Table 2). In the off-pump CABG, 75 proteins showing changes in total (Supplemental Table 3), 35 proteins were found to be up-regulated and 40 proteins were down-regulated (Supplemental Table 4).

Table 2: C-recative protein(CRP), Brain Natriuretic Peptide(BNP) and creatine kinase-MB (CK-MB) concentrations

	BNP(ng/ml)		CRP(mg/dl)		CK-MB (ng/ml)	
	Pre-op	Post-op	Pre-op	Post-op	Pre-op	Post-op
on-pump(n=10)	114.0±13.4	267.0±52.1*	2.3±1.0	16.3±3.3*	59.8±16.1	376.0±41.4*
off-pump(n=10)	98.0±42.3	139.0±42.6*	1.7±0.4	9.8±0.5*	67.4±13.4	195.4±24.9*

aResults are mean±SEM; *P <0.05 post-op versus Pre-op, bPre-op: preoperation; cPost-op: postoperation; dn: the number of sample.

Table 3. The circulating microvesicles in plasma from On-pump and Off-pump CABG before surgery

	A0 (n=10)	B0 (n=10)	P value
Annexin-V	3284.56±1501.43	2887.23±670.91	0.421
CD144	1364.63±444.69	1092.35±136.68	0.067
CD41a	872.38±226.84	843.45±171.65	0.740
CD45	32.87±13.50	26.94±8.73	0.235

aResults are mean±SEM; *P <0.05 A0 versus B0; bA0: On-pump CABG before surgery; cB0: Off-pump CABG before surgery; dn: the number of sample.

Table 4: The circulating microvesicles in plasma from on-pump CABG group

	A0 (n=10)	A1 (n=10)	P value
Annexin-V	3284.56±1501.43	11439.56±7073.92	0.001*
CD144	1364.63±444.69	2268.77±1430.90	0.018*
CD41a	872.38±226.84	1312.83±413.52	0.002*
CD45	32.87±13.50	42.45±12.50	0.150

aResults are mean ± SEM; *P <0.05 A1 versus A0; bA: on-pump CABG group; cA0: Preoperation; dA1: Postoperation; en: the number of sample.

Table 5: The circulating microvesicles in plasma from off-pump CABG group

	B0 (n=10)	B1 (n=10)	P value
Annexin-V	2887.23±670.91	4661.23±1092.85	0.001*
CD144	1092.35±136.68	1439.14±143.9	0.0045*
CD41a	843.45±171.65	1163.57±205.29	0.0132*
CD45	26.94±8.73	38.10±9.71	0.1300

aResults are mean ± SEM; *P <0.05 B1 versus B0; bB: off-pump CABG group; cB0: Preoperation; dB1: Postoperation; en: the number of sample.

Table 6: The circulating microvesicles in plasma from On-pump and Off-pump CABG after surgery

	A1 (n=10)	B1 (n=10)	P value
Annexin-V	11439.56±7073.92	4661.23±1092.85	0.0075*
CD144	2268.77±1430.90	1439.14±143.9	0.0360*
CD41a	2312.83±413.52	1163.57±205.29	0.0260*
CD45	42.45±12.50	38.10±9.71	0.3200

aResults are mean ± SEM; *P <0.05 A1 versus B1; bA1: On-pump CABG after surgery; cB1: Off-pump CABG after surgery; dn: the number of sample.

Supplemental Table 1: Differentially upregulated (>1.20-fold) proteins of cMVs identified by iTRAQ after on-pump CABG surgery

Rank	Accession	Protein Names	Gene Names	Ratio
up1	O75636	Ficolin-3	FCN3	3.135
up2	P10909	Clusterin	CLU	2.941
up3	P00739	Haptoglobin-related protein	HPR	2.841
up4	Q15485	Ficolin-2	FCN2	2.653
up5	P05156	Complement factor I	CFI	2.445
up6	O60814	Histone H2B type 1-K	HIST1H2B	2.336
up7	P04275	von Willebrand factor	VWF	2.336
up8	P27105	Erythrocyte band 7 integral membrane protein	STOM	2.079
up9	P01008	Antithrombin-III	SERPINC1	1.869
up10	Q13201	Multimerin-1	MMRN1	1.859
up11	P02751	Fibronectin	FN1	1.852
up12	Q03591	Complement factor H-related protein 1	CFHR1	1.812
up13	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	1.776
up14	P00488	Coagulation factor XIII A chain	F13A1	1.745
up15	O60706	ATP-binding cassette sub-family C member 9	ABCC9	1.724
up16	P02730	Band 3 anion transport protein	SLC4A1	1.664
up17	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	1.605

up18	P68871	Hemoglobin subunit beta	HBB	1.555
up19	Q9NP78	ATP-binding cassette sub-family B member 9	ABCB9	1.502
up20	P02649	Apolipoprotein E	APOE	1.497
up21	P08519	Apolipoprotein(a)	LPA	1.473
up22	P04114	Apolipoprotein B-100	APOB	1.471
up23	P02452	Collagen alpha-1(I) chain	COL1A1	1.468
up24	Q92954	Proteoglycan 4	PRG4	1.403
up25	Q9NU22	Midasin	MDN1	1.401
up26	P01871	Ig mu chain C region	IGHM	1.397
up27	Q8NG11	Tetraspanin-14	TSPAN14	1.393
up28	P01767	Ig heavy chain V-III region BUT	HV306	1.350
up29	P35527	Keratin, type I cytoskeletal 9	KRT9	1.346
up30	P02679	Fibrinogen gamma chain	FGG	1.337
up31	P02671	Fibrinogen alpha chain	FGA	1.321
up32	Q8NBP7	Proprotein convertase subtilisin/kexin type 9	PCSK9	1.309
up33	P02042	Hemoglobin subunit delta	HBD	1.309
up34	P35858	Insulin-like growth factor-binding protein	IGFALS	1.307
up35	Q9BXR6	Complement factor H-related protein 5	CFHR5	1.304
up36	P02763	Alpha-1-acid glycoprotein 1	ORM1	1.295
up37	Q9Y6R7	IgGFc-binding protein	FCGBP	1.255
up38	P01031	Complement C5	C5	1.250
up39	P07996	Thrombospondin-1	THBS1	1.239
up40	P04003	C4b-binding protein alpha chain	C4BPA	1.232
up41	P05090	Apolipoprotein D	APOD	1.222
up42	P00748	Coagulation factor XII	F12	1.220
up43	P04264	Keratin, type II cytoskeletal 1	KRT1	1.218
up44	P0C0L4	Complement C4-A	C4A	1.215
up45	O14791	Apolipoprotein L1	APOL1	1.208
up46	P69905	Hemoglobin subunit alpha	HBA1	1.202
up47	P02533	Keratin, type I cytoskeletal 14	KRT14	1.200
up48	P02675	Fibrinogen beta chain	FGB	1.200
up49	P18135	Ig kappa chain V-III region HAH	KV312	1.212

Supplemental Table 2: Differentially downregulated (<0.83-fold) proteins of cMVs identified by iTRAQ after On-pump CABG surgery

Rank	Accession	Protein Names	Gene	Ratio
Down1	P09871	Complement C1s subcomponent	C1S	0.828
Down2	P01834	Ig kappa chain C region	IGKC	0.822
Down3	Q15848	Adiponectin	ADIPOQ	0.820
Down4	P36955	Pigment epithelium-derived factor	SERPINF1	0.814
Down5	P01598	Ig kappa chain V-I region EU	KV106	0.813
Down6	P02790	Hemopexin	HPX	0.812
Down7	P27169	Serum paraoxonase/arylesterase 1	PON1	0.807
Down8	P01608	Ig kappa chain V-I region Roy	KV116	0.805
Down9	P01702	Ig lambda chain V-I region NIG-64	LV104	0.804
Down10	P80748	Ig lambda chain V-III region LOI	LV302	0.803
Down11	Q96PD5	N-acetylmuramoyl-L-alanine amidase	PGLYRP2	0.802
Down12	P61626	Lysozyme C	LYZ	0.799
Down13	P07358	Complement component C8 beta chain	C8B	0.794
Down14	P01617	Ig kappa chain V-II region TEW	KV204	0.790
Down15	A0M8Q6	Ig lambda-7 chain C region	IGLC7	0.788
Down16	P27918	Properdin	CFP	0.787
Down17	P02743	Serum amyloid P-component	APCS	0.787
Down18	P02775	Platelet basic protein	PPBP	0.781
Down19	P01034	Cystatin-C	CST3	0.780
Down20	P01042	Kininogen-1	KNG1	0.776
Down21	P0CG05	Ig lambda-2 chain C regions	IGLC2	0.775
Down22	P07357	Complement component C8 alpha chain	C8A	0.773
Down23	P04209	Ig lambda chain V-II region NIG-84	LV211	0.769
Down24	P01611	Ig kappa chain V-I region Wes	KV119	0.765
Down25	P00738	Haptoglobin	HP	0.764
Down26	P06727	Apolipoprotein A-IV	APOA4	0.758
Down27	P0CG06	Ig lambda-3 chain C regions	IGLC3	0.756
Down28	P43652	Afamin	AFM	0.756
Down29	Q14525	Keratin, type I cuticular Ha3-II	KRT33B	0.756
Down30	P02656	Apolipoprotein C-III	APOC3	0.742
Down31	P01714	Ig lambda chain V-III region SH	LV301	0.738

Down32	P25311	Zinc-alpha-2-glycoprotein	AZGP1	0.738
Down33	P01616	Ig kappa chain V-II region MIL	KV203	0.738
Down34	P02768	Serum albumin	ALB	0.732
Down35	P04430	Ig kappa chain V-I region BAN	KV122	0.732
Down36	B9A064	Immunoglobulin lambda-like polypeptide 5	IGLL5	0.716
Down37	P04206	Ig kappa chain V-III region GOL	KV307	0.715
Down38	P02766	Transthyretin	TTR	0.699
Down39	P02747	Complement C1q subcomponent subunit C	C1QC	0.698
Down40	P01625	Ig kappa chain V-IV region Len	KV402	0.689
Down41	P20851	C4b-binding protein beta chain	C4BPB	0.684
Down42	P02647	Apolipoprotein A-I	APOA1	0.629
Down43	P02774	Vitamin D-binding protein	GC	0.618
Down44	P35542	Serum amyloid A-4 protein	SAA4	0.588
Down45	Q9Y6V0	Protein piccolo	PCLO	0.536
Down46	P22352	Glutathione peroxidase 3	GPX3	0.451
Down47	P02655	Apolipoprotein C-II	APOC2	0.413
Down48	P19652	Alpha-1-acid glycoprotein 2	ORM2	0.401
Down49	P07360	Complement component C8 gamma chain	C8G	0.331
Down50	P02776	Platelet factor 4	PF4	0.329
Down51	O00187	Mannan-binding lectin serine protease 2	MASP2	0.304
Down52	P02753	Retinol-binding protein 4	RBP4	0.297

Supplemental Table 3: Differentially upregulated (>1.20-fold) proteins of cMVs identified by iTRAQ after Off-pump CABG surgery

Rank	Accession	Protein Names	Gene	Ratio
up1	P02745	Complement C1q subcomponent subunit A	C1QA	1.779
up2	P02452	Collagen alpha-1(I) chain	COL1A1	1.672
up3	P04259	Keratin, type II cytoskeletal 6B	KRT6B	1.587
up4	P19652	Alpha-1-acid glycoprotein 2	ORM2	1.582
up5	P27105	Erythrocyte band 7 integral membrane protein	STOM	1.565
up6	P20851	C4b-binding protein beta chain	C4BPB	1.558
up7	Q03181	Peroxisome proliferator-activated receptor delta	PPARD	1.548
up8	P02776	Platelet factor 4	PF4	1.497
up9	Q8NG11	Tetraspanin-14	TSPAN14	1.479
up10	P02746	Complement C1q subcomponent subunit B	C1QB	1.477
up11	P02655	Apolipoprotein C-II	APOC2	1.418
up12	O60706	ATP-binding cassette sub-family C member 9	ABCC9	1.368
up13	P36980	Complement factor H-related protein 2	CFHR2	1.309
up14	Q96PD5	N-acetylmuramoyl-L-alanine amidase	PGLYRP	1.295
up15	O60814	Histone H2B type 1-K	HIST1H2B	1.282
up16	P36955	Pigment epithelium-derived factor	SERPINF1	1.282
up17	P02775	Platelet basic protein	PPBP	1.274
up18	P27918	Properdin	CFP	1.266
up19	Q13201	Multimerin-1	MMRN1	1.359
up20	P00488	Coagulation factor XIII A chain	F13A1	1.359
up21	Q8NBP7	Proprotein convertase subtilisin/kexin type 9	PCSK9	1.342
up22	P09871	Complement C1s subcomponent	C1S	1.325
up23	P02749	Beta-2-glycoprotein 1	APOH	1.314
up24	P04003	C4b-binding protein alpha chain	C4BPA	1.258
up25	Q15848	Adiponectin	ADIPOQ	1.235
up26	Q9NP78	ATP-binding cassette sub-family B member 9	ABCB9	1.235
up27	P02656	Apolipoprotein C-III	APOC3	1.225
up28	P01781	Ig heavy chain V-III region GAL	HV32	1.225
up29	P02675	Fibrinogen beta chain	FGB	1.221
up30	Q9Y6V0	Protein piccolo	PCLO	1.220
up31	P02774	Vitamin D-binding protein	GC	1.212
up32	Q9NU22	Midasin	MDN1	1.212
up33	P01860	Ig gamma-3 chain C region	IGHG3	1.203
up34	Q15485	Ficolin-2	FCN2	1.203
up35	P0C0L4	Complement C4-A	C4A	1.202

Supplemental Table 4: Differentially downregulated (<0.83-fold) proteins of cMVs identified by iTRAQ after Off-pump CABG surgery

Rank	Accession	Protein Names	Gene Names	Ratio
Down1	P02747	Complement C1q subcomponent subunit C	C1QC	0.831
Down2	P04264	Keratin, type II cytoskeletal 1	KRT1	0.828
Down3	Q92954	Proteoglycan 4	PRG4	0.824
Down4	P02743	Serum amyloid P-component	APCS	0.824
Down5	P01604	Ig kappa chain V-I region Kue	KV112	0.822
Down6	P23142	Fibulin-1	FBLN1	0.820
Down7	P00751	Complement factor B	CFB	0.818
Down8	P01598	Ig kappa chain V-I region EU	KV106	0.818
Down9	P13671	Complement component C6	C6	0.809
Down10	P01617	Ig kappa chain V-II region TEW	KV204	0.808
Down11	P06396	Gelsolin	GSN	0.797
Down12	P02753	Retinol-binding protein 4	RBP4	0.797
Down13	P10643	Complement component C7	C7	0.796
Down14	P15814	Immunoglobulin lambda-like polypeptide 1	IGLL1	0.792
Down15	P00747	Plasminogen	PLG PE	0.787
Down16	P26927	Hepatocyte growth factor-like protein	MST1	0.777
Down17	P01768	Ig heavy chain V-III region CAM	HV307	0.776
Down18	P01611	Ig kappa chain V-I region Wes	KV119	0.776
Down19	P05160	Coagulation factor XIII B chain	F13B	0.773
Down20	P05546	Heparin cofactor 2	SERPIND1	0.756
Down21	P00738	Haptoglobin	HP	0.743
Down22	P02533	Keratin, type I cytoskeletal 14	KRT14	0.742
Down23	P04208	Ig lambda chain V-I region WAH	LV106	0.738
Down24	P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	0.732
Down25	P02760	Protein AMBP	AMBP	0.729
Down26	P02751	Fibronectin	FN1	0.728
Down27	P01833	Polymeric immunoglobulin receptor	PIGR	0.715
Down28	P00734	Prothrombin	F2	0.689
Down29	P04275	von Willebrand factor	VWF	0.669
Down30	P13645	Keratin, type I cytoskeletal 10	KRT10	0.663
Down31	P08519	Apolipoprotein(a)	LPA	0.652
Down32	P43652	Afamin	AFM	0.651
Down33	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	0.650
Down34	P22792	Carboxypeptidase N subunit 2	CPN2	0.776
Down35	O00187	Mannan-binding lectin serine protease 2	MASP2	0.776
Down36	P07360	Complement component C8 gamma chain	C8G	0.773
Down37	P02730	Band 3 anion transport protein	SLC4A1	0.756
Down38	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	0.743
Down39	P04114	Apolipoprotein B-100	APOB	0.742
Down40	P22352	Glutathione peroxidase 3	GPX3	0.738

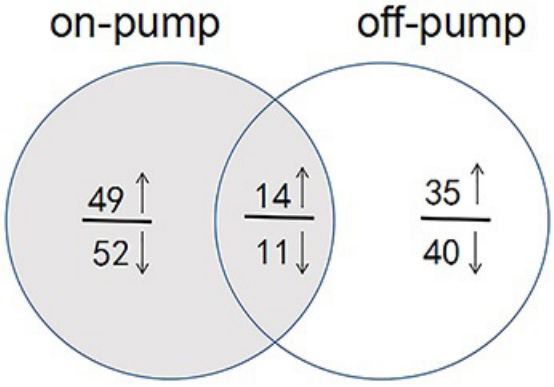

Figure 1: Venn diagram showed that the number of total differentially expressed proteins in on-pump and off-pump CABG group were identified by tandem mass spectrometry (MS/MS). The overlapping regions indicated the number of sharing proteins. The number above or below the horizontal line in each portion indicated the number of up- or down-regulated proteins respectively

4.4. Functional Classification of the Differentially Expressed Proteins

The screened proteins were functionally catalogued by GO in two different groups. All of the results showed that the related proteins were associated with the process of change during CABG with/without the use of CPB. Most of the proteins were responsed to inflammation and coagulation. In the off-pump CABG group, as shown in (Figure 2), the proteins were involved in biological process including coagulation, Fc receptor-mediated stimulatory signaling pathway, regulation of response to stimulus, complement activation, response to stimulus, vesicle-mediated transport, response to stress, complement activation, Fc receptor signaling pathway, inflammatory response, cell communication, and regulation of blood coagulation, and so on. The identified proteins were separated according to the cellular component, include the cell part, the extracellular region part, the cellular component, and the membrane-bound organelle. The molecular function of the proteins was classified as molecular function, protein binding, and enzyme regulator activity etc. (Figure 3) showed the changes in the on-pump CABG group, and similar to the off-pump CABG group, the proteins were involved in biological process including response to stimulus, Fc-gamma receptor signaling pathway, complement activation, alternative pathway, vesicle-mediated transport, acute inflammatory response, vesicle-mediated transport, platelet activation, and cell communication. The identified proteins according to the cellular component included the membrane, the membrane-bound organelle, the membrane-bound vesicle, and the cellular component. Molecular function of the proteins was classified as molecular function, binding, phospholipid binding, active transmembrane transporter activity, substrate-specific transporter activity etc. Enriched pathways participated via the different expressed proteins.

To gain a deeper understanding of the proteins involved in the biological mechanisms of cMVs, a publicly available KEGG pathway database was used to provide biologists with excellent resources with respect to on- and off-pump. Approximately 50 KEGG pathways were detected (data not shown). Among these proteins, VWF, SERPIND1, C1-8, F12, and F13 were found in the complement and coagulation cascade pathway; and VWF was found in the platelet activation pathway. The levels of these proteins were significantly increased after the surgery in the on-pump CABG group, but there was no difference in the off-pump CABG group (Figure 4).

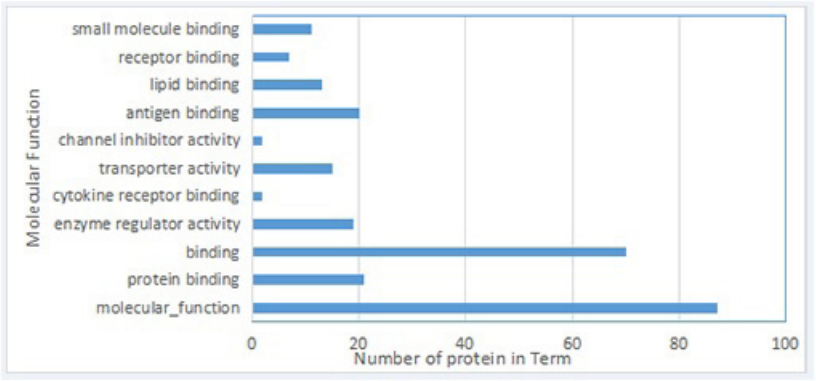

Figure 2: Classification of identified proteins in off-pump
Figure 2.1: Cellular components (CCs)

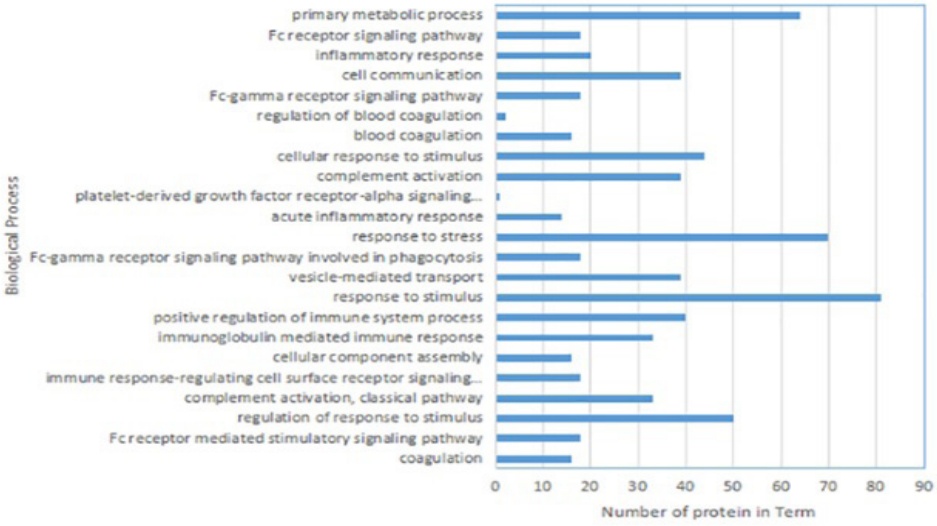

Figure 2.2: Molecular functions (MFs)

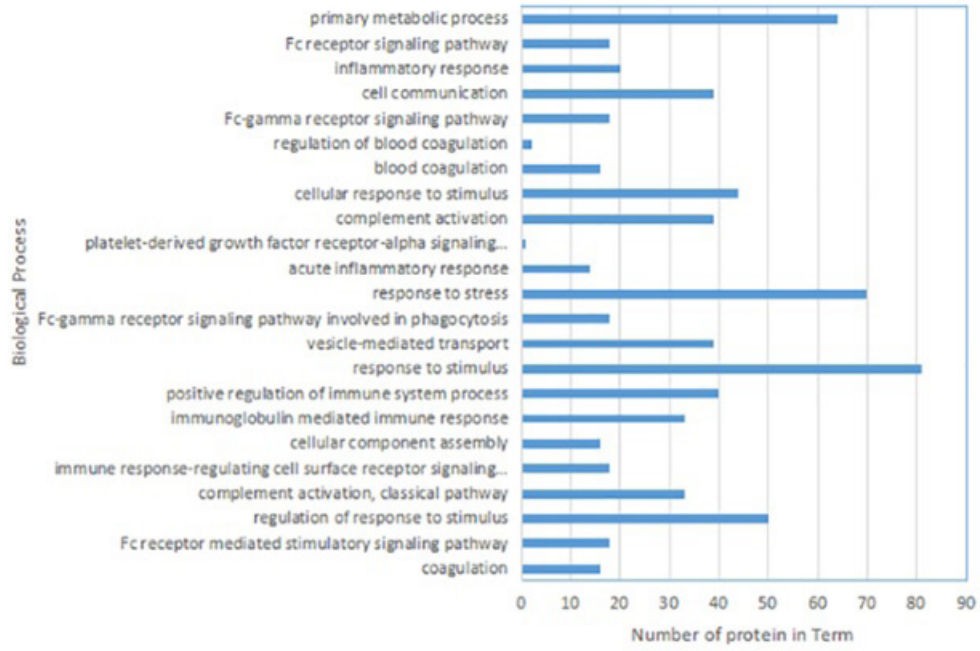

Figure 2.3: The biological processes (BPs) of the differentially expressed proteins were selected by GO database

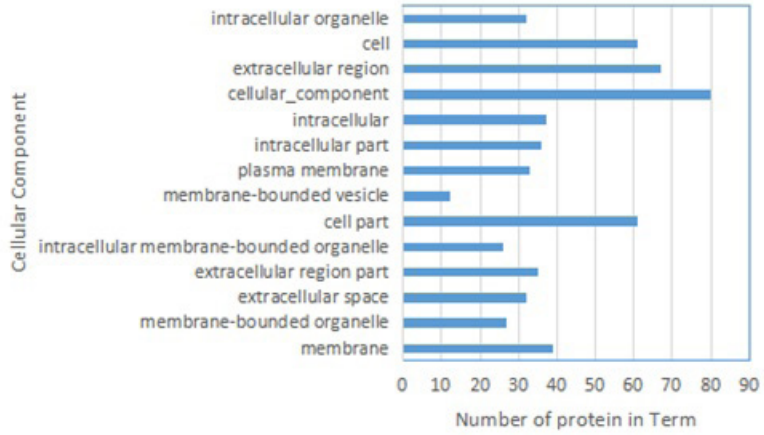

Figure 3: Classification of identified proteins in on-pump

Figure 3.1: Cellular components (CCs)

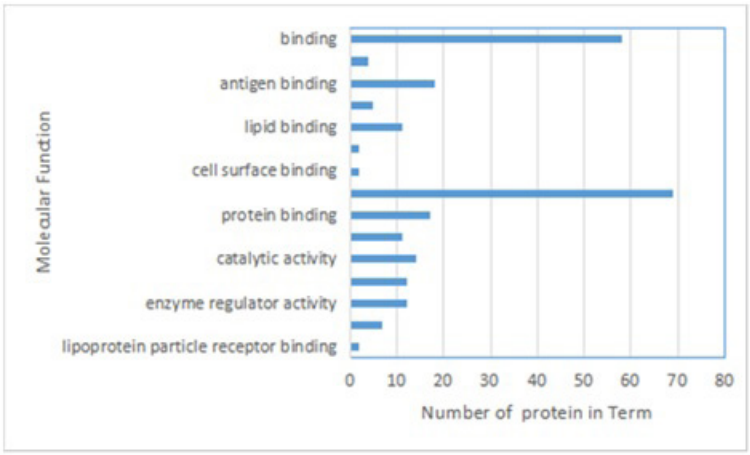

Figure 3.2: Molecular functions (MFs)

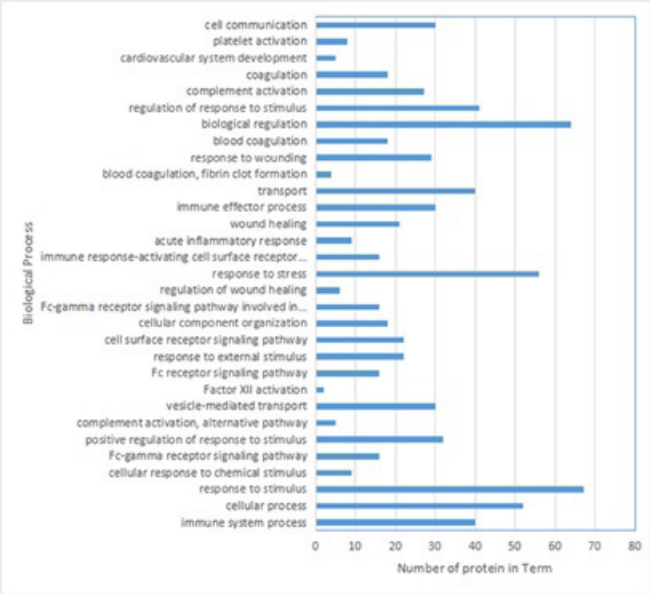

Figure 3.3: The biological processes (BPs) of the differentially expressed proteins were selected by GO database

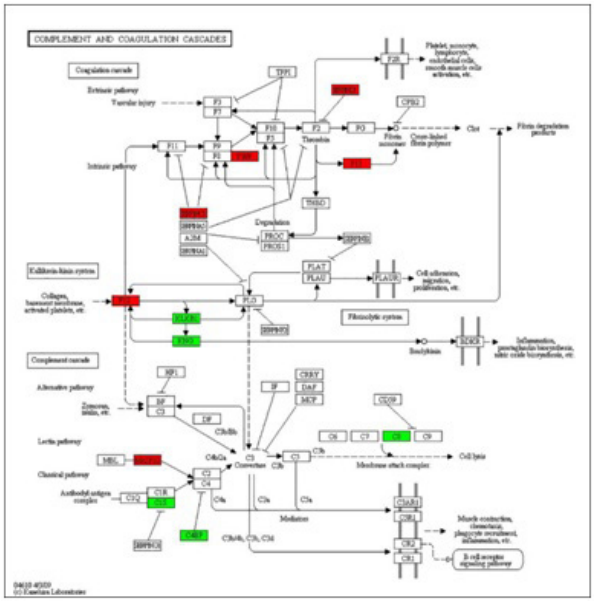

Figure 4.1: Differentially expressed proteins in Platelet activation pathway in the on-pump CABG group. Red text denotes up-regulated proteins. Green text denotes down-regulated

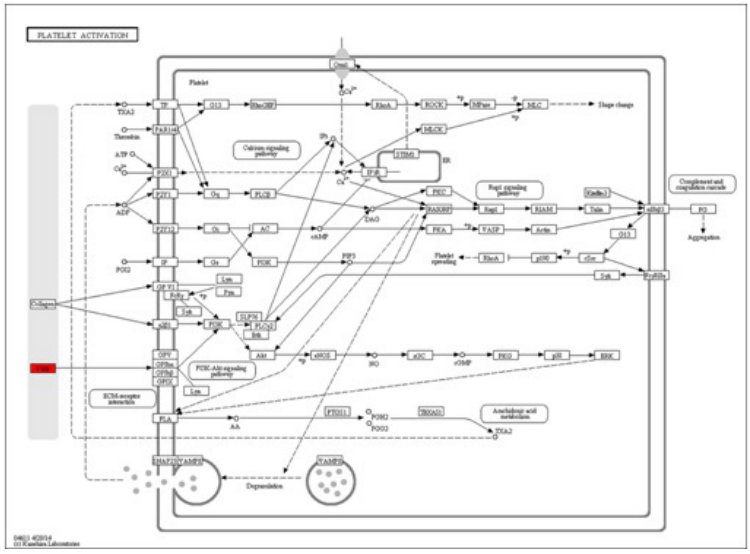

Figure 4.2: Differentially expressed proteins in Platelet activation pathway in the on-pump CABG group. Red text denotes up-regulated proteins. Green text denotes down-regulated

5. Discussion

Our study demonstrated the simultaneous level changes of Brain Natriuretic Peptide (BNP), C-Reactive Protein (CRP), Myoglobin (Mb), and Circulation microvesicles in CABG patients. We showed that the changing of circulation microvesicles in postoperative patients was in line with the markers of inflammation, platelet activation and myocardical dysfunction. Postoperatively all studied parameters, including microvesicles, BNP, Mb and CRP, increased statistically higher in both on-pump and off-pump. In contrast to on-pump surgery, off-pump approach less increased the levels of these markers. Simultaneously, the microvesicles was performed by flow cytometry, positively expressed Phosphatidylserine (PS), endothelial CD144 marker, platelet CD41a marker, and white blood cell CD45 marker.

Many factors are responsible for the inflammatory response, including surgical trauma, cardiopulmonary bypass, thrombin activation and myocardial injury [18-23]. At present, there are no specific markers and no obvious difference to the inflammatory response in patients undergoing on-pump and off-pump. According to previous researches, CRP, IL-6 and IL-8 induced by the surgical trauma are associated with the level of inflammatory response in patients [1, 11, 23]. In addition, the complement system and fibrinolysis are also activated [24-25]. In our study, we demonstrated that the level of CRP significantly increased in postoperative patients, and was greater after on-pump group. Myocardical dysfunction could not avoid and directly effected the mortality of postoperative patients. Based on the previous studies, comparing to the on-pump, the incidence of myocardial infarction was significantly less in off-pump patients [5, 19, 26]. In this study, BNP and Mb also have shown statistically increased in on-pump patients. Here, our result is consistent with the reported. Surgical trauma always causes a series of endothelial and soft tissue injury, which could lead to coagulopathy. However, it is difficult defined the extent of coagulation [27]. Microvesicles increasingly released under stress conditions, including surgery, inflammation and so on. They are considered as a potential means of intercellular communication, because they are capable of transferring various substances between cells and organs, such as proteins, mRNA, and miRNA. Earlier studies have suggested microvesicles in blood are derived from many kinds of cells, including platelets, erythrocytes, granulocytes, monocytes, lymphocytes, and endothelial cells. In physiology, platelet microvesicles are mostly detected [28-29]. In the process, the main change was the outward translocation of Phosphatidylserine (PS), which could be connected with Annexin-V [30]. This phenomenon also saw in this study, almost of microvesicles positively expressed PS. In inflammatory response, microvesicles was involved in many different mechanisms, for example, by stimulating the expression of pro-inflammatory genes in endothelial cells, leading to the production of cytokines and leukocyte-endothelial cell adhesion molecules in vitro, or by exposing complement components (C1q, C3, C4, and C5) [28]. Consequently, iTRAQ-based proteomic analysed further cMVs, we detected a total of 201 different expressed proteins in the two groups. These up-regulated proteins showed to participate in multiple biological processes (such as single-organism metabolic process, regulation of vesicle-mediated transport, and development process), and signaling pathways including cytokine-cytokine receptor interaction, the chemokine signaling pathway, ECM-receptor interaction, complement and coagulation cascades, and platelet activation. Based on the above data, we proposed the level of cMVs were associated with the injury degree in CABG. And circulating microvesicles paticipated and played a key role in the inflammatory response and coagulopathy in Coronary Artery Bypass Graft Surgery.

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