Original Paper

Peptidomic Analysis of Amniotic Fluid for Identification of Putative Bioactive Peptides in Ventricular Septal Defect

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Key Words

Ventricular septal defect • Amniotic fluid • Peptidomic analysis • Liquid chromatography tandem mass spectrometry

Abstract

Background: Ventricular septal defect (VSD) is one of the most common congenital heart diseases and to date the role of peptides in human amniotic fluid in the pathogenesis of VSD have been rarely investigated. **Methods:** To gain insight into the mechanisms of protein and peptides in cardiovascular development, we constructed a comparative peptidomic profiling of human amniotic fluid between normal and VSD fetuses using a stable isobaric labeling strategy involving tandem mass tag reagents, followed by nano liquid chromatography tandem mass spectrometry. **Results:** We identified and quantified 692 non-redundant peptides, 183 of which were differentially expressed in the amniotic fluid of healthy and VSD fetuses; 69 peptides were up regulated and 114 peptides were down regulated. These peptides were imported into the Ingenuity Pathway Analysis (IPA) and identified putative roles in cardiovascular system morphogenesis and cardiogenesis. **Conclusion:** We concluded that 35 peptides located within the functional domains of their precursor proteins could be candidate bioactive peptides for VSD. The identified peptide changes in amniotic fluid of VSD fetuses may advance our current understanding of congenital heart disease and these peptides may be involved in the etiology of VSD.

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Published by S. Karger AG, Basel

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Introduction

Ventricular septal defect (VSD) is one of the commonest congenital malformations of the heart, accounting for up to 40% of all cardiac anomalies [1]. The incidence of VSD is approximately 3.94 per 1000 live births [2, 3], and congenital heart disease is causing society a heavy psychological and economic burden. Due to the progress of new molecular biology techniques, the past decade has witnessed spectacular development in elucidation of the molecular mechanisms of heart formation. In particular, several transcription factors (TFs) such as NKX2.5 [4], GATA4 [5], TBX5 [5] and HOMEZ [6] have been identified as being essential for heart. However, little is known about the proteomic and peptidomic studies related to the pathology of congenital heart disease (CHD). Besides, changes in genomic and transcriptomic approaches may not quantitatively correlate well with the expression of proteins [7, 8] and cannot characterize post-translational modifications (PTMs) which are prominently involved in modulating many biological processes. Cardiovascular proteomics dramatically broadening our knowledge on the complex myocardial physiology and pathophysiological states with an enormous potential to rapidly advance the identification of disease mechanisms.

As we know, echocardiography is the mainstay of modern diagnosis of ventricular septal defect, but the diagnosis of congenital heart disease in fetuses by echocardiography depends highly on the examiner's skills and experiences, which may cause misdiagnosis. Reliable objective diagnostic biomarkers for VSD in early pregnancy is still needed for early detection and for predicting prognosis after birth, which may provide useful information to aid prenatal counselling and choice of delivery method. A greater understanding of the molecular basis of cardiac function will help guide the development of novel diagnostic and therapeutic strategies. A recent proteomic study of plasma protein in CHDs [9] was composed of multiple kinds of CHD. VSD is not only a common isolated cardiac malformation, but also an intrinsic component of several complex malformations. In the present study we focused on patients for whom VSD is the predominant form of congenital heart disease.

Recent technical advances in proteomics and associated methodologies will allow us to investigate VSD to an unprecedented depth. This is the first molecular biology and bioinformatics representation of peptidomic analysis on VSD. Peptidomics is an emerging branch of proteomics that investigates endogenously produced protein fragments [10]. Peptidomics can present a dynamic view of the health status of a tissue or sample, and they may provide insight into the complex and fluid interaction of activators, inhibitors, proteases, and protein substrates. In the past, intact proteins were considered as the functional units *in vivo*. In reality, most proteins experience proteolytic processing such as activation or degradation by enzymes [11]. Therefore, in most cases, endogenous peptides can interact directly with the cellular targets, producing a functional effect.

Amniotic fluid (AF) fills the amniotic cavity that is lined by amnion epithelial cells that are of fetal origin and hence reflect the pathological changes of the fetus [12, 13]. Vesce et al. [12] found that ampicillin administration may directly decrease amniotic IL-6 and PGE2 release through management of bacterial and nonbacterial inflammatory complications of pregnancy by prostanoid and cytokine interaction. In addition, Morabito et al. [14] utilize mesenchymal stem cells from human amniotic fluid as a osteogenic model to study *in-vitro* cell responses to calcitonin, which provide new insight in cell therapies and pharmacological use of these molecules. The composition of AF is modified during pregnancy, and its protein profile reflects the physiological and pathological changes that affect both the fetus and the mother. In the present study, we conducted a comprehensive peptidomic analysis of human AF in healthy and VSD fetuses using tandem mass tag (TMT) labelling coupled with nano LC-MS/MS in hope to explore bioactive peptides during heart development and in the pathogenesis of VSD.

Materials and Methods

Materials

Novex NuPAGE 10% Bis-Tris Gels were obtained from Life Technologies (Rockford, USA); Coomassie Brilliant Blue-G250 and PageRuler prestained protein ladder were purchased from Thermo Fisher Scientific (Rockford, USA); HPLC-grade acetonitrile (ACN) was obtained from Tedia (Fairfield, OH, USA); HPLC-grade formic acid (FA) was purchased from Sigma/Fluka (Switzerland).

Sample collection

Amniotic fluid (AF) were obtained from pregnant women at 23–25 weeks of gestation who underwent amniocentesis and routine blood test at the second trimester of pregnancy in Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University from April 10th to August 15th, 2015. Samples were collected with written consent and ethics board approval from Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University. VSD fetuses were identified by a characteristic echo dropout in the ventricular septum in two-dimensional echocardiography (Fig. 1A), a typical systolic coloured flame crossing the septum (Fig. 1B). Fetuses with associated complex extra-cardiac anomalies (such as fetal edema and renal dysplasia) and chromosome abnormalities (such as Trisomy 21 and Trisomy 18) were excluded from the study.

The control group consisted of women with natural, singleton pregnancies who underwent termination, and whose screening ultrasound reports were normal during pregnancy. Samples were thus normal controls (n = 3) or VSD group members (n = 3). Samples were matched for gestational age and

Fig. 1. (A) Echocardiogram showing a small fetal ventricular septal defect (indicated by a white arrow). (B) Colour Doppler echocardiographic examination of blood shunting in a VSD fetus.

Table 1. Clinical features of VSD patient and control groups

Number	Age	Gestational week	T-P-A-L	BPD (cm)	FL (cm)	AFV (cm)
NC-1	34	24 + 5	1-0-2-1	4.6	2.7	4.2
NC-2	32	22 + 6	1-0-1-1	5	3.1	5.45
NC-3	29	23 + 6	0-0-0-0	5.41	4.26	4.4
VSD-1	29	24 + 2	0-0-1-0	5.5	3.8	4
VSD-2	33	23 + 1	1-0-1-1	5.8	4	4.5
VSD-3	27	24 + 5	0-0-0-0	5.39	3.92	4.5

T: Term infants, P: Preterm infants, A: Abortuses, L: Living children,
BPD: biparietal diameter; FL: femur length AFV: amniotic fluid volume

maternal age, and the detailed characteristics of patient and control groups are listed in Table 1. Samples were collected, stored on ice during transport to the laboratory (up to 1 h), centrifuged (1000 g, 10 min, 4°C) to remove cellular debris, protease inhibitor mixture (Complete Mini EDTA-free, Roche, Basel, Switzerland) was added and supernatants were stored at -80°C until needed.

Sample preparation and ultrafiltration

Samples were centrifuged at 12,000 g at 4°C for 15 min after thawing on ice, ACN (20% (v/v)) was added to supernatants, and samples were briefly vortexed and incubated for 20 min at room temperature. Centrifugal concentrators (Amicon Ultra-15, Millipore) with a molecular weight cut-off (MWCO) of 10 kDa were washed with 0.5 ml H₂O and samples were passed through the filters according to the manufacturer's recommendations. The flow-through containing peptides was recovered and lyophilized, and the concentration of peptides/proteins in the supernatant was measured using the BCA protein assay (Pierce, Rockford, IL, USA).

NuPAGE Bis-Tris gel electrophoresis

Extracted peptides (35 µg) were dissolved in NuPAGE Bis-Tris LDS sample buffer, heated at 70°C for 10 min, and each sample was loaded onto a single lane. Separation was performed in NuPAGE MES running buffer and proteins were visualized using Coomassie Brilliant Blue staining as previously described [15]. The staining revealed that almost all high molecular weight proteins have been removed (Fig. 2).

Peptide desalting and TMT labelling

Samples containing 100 µg peptides were reduced with 10 mM DTT at 60°C for 1 h, alkylated with 55 mM iodoacetamide (IAA) for 45 min at room temperature, desalted and dried in vacuo (Speed Vac, Eppendorf). AF peptides were labelled with isobaric tags based on total peptide amount at room temperature for 1 h. TMT labelling was performed according to manufacturer's instructions (TMT 6-plex Label Reagent, Thermo Scientific). TMT results were generated from analysis of isobaric tag combinations. Three normal control AF samples were labelled with reagents 126, 127 and 128, and three VSD AF samples were labelled with reagents 129, 130 and 131.

Mass spectrometry analysis, peptide identification and quantification

Peptides were analyzed by nanoflow LC (Easy-nLC, ThermoFisher Scientific Inc., San Jose, CA) coupled with an LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific Inc.). Reverse-phase separation of peptides was performed using buffer A (2% ACN, 0.5% acetic acid) and buffer B (80% ACN, 0.5% acetic acid) with a gradient. Eluted peptides were electrosprayed at a voltage of 1.8 kV into the mass spectrometer which was configured to collect high resolution ($R = 60,000$ at m/z 400) broadband mass spectra (m/z 375–1800) using the lock mass feature for the polydimethylcyclsiloxane ion generated in the electrospray process (m/z 445.12002). The 20 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 35%. The most intense product ion from the MS2 step was selected for higher energy collision-induced dissociation (HCD) MS3 fragmentation.

Extracted MS/MS spectra were searched against the database containing 20194 human protein sequences (released May 2015) using in-

Fig. 2. NuPAGE Bis-Tris gel electrophoresis and Coomassie Brilliant Blue staining of amniotic fluid proteins. Different fractions exhibited different protein profiles, and almost all high molecular weight proteins were removed by ultrafiltration. (M=marker, the unit is kiloDalton. 1: untreated amniotic fluid, 2: purified peptides of healthy normal control fetus, 3: purified peptides of ventricular septal defect fetus).

house PEAKS software (version 7.0, Bioinformatics Solutions, Waterloo, Canada). The fusion target-decoy approach was used for estimation of the false discovery rate (FDR) and was set at $\leq 1\%$ ($-10 \log P \geq 20.0$) at both protein and peptide levels. Peptides were considered positively identified only if peptide peaks were present in at least two spectra per sample.

Relative quantification of the AF peptidome was performed using the TMT labelling approach in the PEAKS Q module. Feature detection was performed separately on each sample using the expectation-maximization algorithm. Features of identical peptides from different samples were aligned using high-performance retention time alignment algorithm [16]. Peptides and proteins were considered to be significantly altered between AF samples when the p-value was < 0.05 and the fold change was > 1.5 . Identified results were included during the last step of TMT labelling quantification.

Bioinformatics analysis

The isoelectric point (pI) of each peptide was calculated using the pI/Mw online tool (http://web.expasy.org/compute_pi/). Pathway and gene ontology (GO) analyses (<http://geneontology.org>) were carried to determine potential physiological functions. GO results included three categories; cellular component, biological process and molecular function. The threshold of significance was defined by the P-value and FDR. To further explore the significance of differentially expressed peptides and their precursor proteins, they were imported into the Ingenuity Pathway Analysis (IPA) Software v7.1 (Ingenuity Systems, Mountain View, CA) for pathway analysis as described previously [17]. This software can also be used for biochemical, biologic, and molecular functions. The identified proteins were mapped to associated network functions that were generated from existing literature from the Ingenuity Systems Knowledge Base. The MEROPS database (<http://merops.sanger.ac.uk>) was used to seek out candidate enzymes for given cleavage sites [18].

Statistical Analysis

SPSS 15.0 software (SPSS Inc, Chicago, IL) were used for statistical analysis. T-test was used to compare values between control and VSD groups, and statistical significance was defined as p of less than 0.05.

Results

Identification of differentially expressed peptides in human amniotic fluid

A total of 63 peptides significantly differentially expressed (fold change > 2.0) were listed in Table 2 and they were visualized using heat maps (Fig. 3A and 3B). Each of the six columns represents healthy control or VSD groups, and the magnitude of peptide expression is coloured according to the heat map scale. We identified and quantified 692 non-redundant peptides, 183 of which were differentially expressed in the amniotic fluid of healthy and VSD fetuses. Raw data have been uploaded to the public database: <http://58.193.178.2/datacenter/>.

Details of peptides identified in human amniotic fluid

Following analysis of the differentially expressed peptides identified in human amniotic fluid, we found that both molecular weight (MW) and isoelectric point (pI) were spread over a wide range, but most peptides were distributed below 1000 Da in molecular weight and 5.0-7.0 in isoelectric point (Fig. 4A and 4B). The relative distribution of pI versus MW was also investigated (Fig. 4C), and the MW of all identified peptides was below 3 kDa, confirming the isolated peptides were of adequate purity.

Cleavage site patterns of identified endogenous peptides

We combined peptidome analysis of human amniotic fluid by LC-MS/MS with bioinformatics analysis of cleavage sites at the N- and C-termini. Four cleavage sites were investigated for each identified up-regulated and down-regulated peptide (Fig. 4D and 4E), respectively. In up-regulated group the four dominant amino acids of the four cleavage sites

Table 2. Part of the peptides that are differently expressed in in amniotic fluid of VSD fetuses

Accession	Protein	Peptide	MW (KDa)	P-value	Fold change
Peptides up-regulated in amniotic fluid of VSD fetuses (>2 folds)					
Q96CD0	FBXL8	VALRRRHPLAV	1344.63	7.56E-04	+∞
P19544	WT1	ALLPAVPSL	880.1	4.61E-02	4.85
Q9Y6R4	M3K4	GFVLNRS	878.99	3.42E-02	4.69
Q96JH8	RADIL	GDRLI	572.66	3.46E-02	3.85
Q12923	PTN13	GDRLI	572.66	3.46E-02	3.85
P39059	COFA1	PVRPH	604.71	4.14E-02	3.47
Q7Z5P9	MUC19	CGSQCTCQ	828.93	4.30E-03	3.24
Q99466	NOTC4	YSGPTCH	763.82	9.75E-03	2.83
P60508	SYCY2	SSCSTR	639.68	4.24E-02	2.76
Q70EL4	UBP43	SRFLLAL	819.01	1.15E-02	2.73
Q5TC82	RC3H1	GGDNND	677.58	8.39E-03	2.68
Q9UPS6	SET1B	AGSPELSP	853.93	1.38E-02	2.62
Q8NEL9	DDHD1	ASSSGTR	664.67	4.56E-02	2.62
Q8IZU2	WDR17	KTGFH	588.66	7.59E-03	2.32
Q86V15	CASZ1	SPLATT	588.66	7.59E-03	2.32
Q15303	ERBB4	DSSSCVRA	823.88	3.38E-03	2.31
Q86YD3	TMM25	PAALRHTLLL	1104.36	1.71E-02	2.25
Q8WZ42	TITIN	DGAALSPSPN	927.97	3.83E-02	2.21
Q5VW99	TAF3	APPLVLPP	803.01	1.87E-02	2.16
Q03001	DYST	SMTSGL	594.68	4.27E-02	2.08
Q8WZ42	TITIN	NQTVL	573.65	5.40E-02	2.04
Q6ZSN1	YI023	QSPPRGR	796.88	4.37E-02	2.04
P25940	CO5A3	DPGPPGAPGIPG	1128.25	3.74E-02	2.04
Peptides down-regulated in amniotic fluid of VSD fetuses (>2 folds)					
Q13467	FZD5	TGPPGPAA	666.73	4.83E-02	-2.01
Q6UXD5	SE6L2	SPASPGPLGPE	1105.21	4.09E-02	-2.05
Q8WZ42	TITIN	INETLELL	944.09	2.63E-03	-2.05
Q5VV17	OTUD1	AVPAAK	555.68	6.04E-03	-2.09
P13942	COBA2	AKGDEGTRGFNGPPGPIGLQGLPGPS	2476.73	5.70E-02	-2.13
Q6ZRS2	SRCAP	PPLAPVV	691.87	1.50E-02	-2.15
Q58EX2	SDK2	AEGSWPL	758.83	2.46E-02	-2.20
P42226	STAT6	VERDQPLPTP	1151.28	4.07E-02	-2.21
Q6V0L0	CP26C	FHPLT	613.71	4.15E-02	-2.23
Q9Y520	PRC2C	PPPLTT	624.73	3.72E-02	-2.28
Q9C0A1	ZFH2	ENFGF	612.64	2.66E-02	-2.29
P02462	CO4A1	GVTGLVGIPGPPGIP	1330.59	3.41E-02	-2.30
Q8IUR6	CRERF	HATPALP	705.81	3.49E-02	-2.31
Q8WZ42	TITIN	GPMLH	553.68	6.24E-03	-2.37
Q13686	ALKB1	AIFLLGGL	803.01	3.71E-02	-2.53
Q9NQ74	EXOS5	AQAASQH	711.73	4.51E-02	-2.57
Q99698	LYST	HVGTFSH	783.84	4.15E-02	-2.62
O14647	CHD2	RRPVP	623.76	6.22E-03	-2.65
Q8WZ42	TITIN	ASHSSTK	716.75	2.17E-02	-2.75
Q2UY09	COSA1	FEIFH	691.78	4.40E-02	-2.78
O43451	MGA	FELFH	691.78	4.40E-02	-2.78
Q5SXM2	SNPC4	VVRER	657.77	1.44E-02	-2.81
Q99700	ATX2	VPVSPGV	653.78	4.87E-02	-3.00
O75844	FACE1	VFLLL	603.8	4.90E-02	-3.13
Q9UI33	SCNBA	VFILI	603.8	4.90E-02	-3.13
Q8NGY1	O10Z1	VFIII	603.8	4.90E-02	-3.13
Q9NYW6	TA2R3	VFLIL	603.8	4.90E-02	-3.13
Q63HN8	RN213	VFLLI	603.8	4.90E-02	-3.13
Q7Z404	TMC4	VFILL	603.8	4.90E-02	-3.13
P46098	5HT3A	VFLII	603.8	4.90E-02	-3.13
Q9Y3Q4	HCN4	SPPPPQV	720.82	9.48E-03	-3.31
Q8NDA2	HMCN2	FVLLI	603.8	4.91E-02	-3.43
Q6ZMW3	EMAL6	FVILL	603.8	4.91E-02	-3.43
Q58EX2	SDK2	FVLII	603.8	4.91E-02	-3.43
O75023	LIRB5	FVLLL	603.8	4.91E-02	-3.43
Q86UX2	ITIH5	FVILI	603.8	4.91E-02	-3.43
Q96BF3	TMIG2	LFVDP	589.69	4.42E-02	-3.58
Q13428	TCOF	IFVDP	589.69	4.42E-02	-3.58
Q99583	MNT	HVLQGGP	706.8	2.90E-02	-3.62
Q9Y4A5	TRRAP	EFLMPK	763.95	1.09E-03	-3.68

(C-terminal amino acid of the preceding peptide, N-terminal amino acid of the identified peptide, C-terminal amino acid of the identified peptide, N-terminal amino acid of the subsequent peptide) were glycine (G), glycine (G), leucine (L), glycine (G), while in down-

Fig. 3. Distribution of differentially expressed peptides between normal controls and the VSD group visualized using a heat map coloured based on $\log_{10}$ values. (A): Down-regulated peptides; (B): up-regulated peptides. The extent of peptide abundance is coloured according to the heat map scale (NC: normal control, VSD: ventricular septal defect, over-expression: red, low expression: green, and no expression: yellow).

Fig. 4. Characteristics of amniotic fluid peptides identified by LC-MS/MS. (A) Molecular weight (MW). (B) Isoelectric point (pI). (C) Scatter plot of MW versus pI. (D) Distribution of the four cleavage sites in the identified up-regulated peptides. (E) Distribution of the four cleavage sites in the identified down-regulated peptides. (F) Peptides shared the same precursor protein

Fig. 5. Gene ontology (GO) and subcellular locations of differentially expressed peptides and their precursor proteins. (A) GO analysis of the biological process category. (B) GO analysis of the cellular component category. (C) GO analysis of the molecular function category. (D) Mapping of differentially expressed proteins to canonical signalling pathways by Ingenuity Pathway Analysis (IPA).

regulated group the four most amino acids were glycine (G), glycine (G), proline (P), proline (P). There were more than one peptides derived from the same precursor protein. The precursor proteins were listed in Fig. 4F with titin having largest number of related peptides.

Annotation of peptide precursors and canonical pathway of peptides

Pathway and gene ontology (GO) analyses were carried out to determine potential roles for the identified peptides and their precursor proteins. The most common biological process, cellular component, and molecular function categories were listed in Fig. 5A-C, respectively. As amniotic fluid is composed of metabolite of various organs, and results also involved in multiple organs. Biological processes particularly involved in cardiogenesis were cell morphogenesis involved in differentiation, cardiovascular system development and circulatory system development. Precursor proteins significantly differentially expressed in the AF of the VSD group were mapped against canonical pathways, and those with the highest statistical significance were plotted as a bar chart (Fig. 5D).

Functional clustering using Ingenuity Pathway Analysis

Differentially expressed proteins were investigated using IPA software (Qiagen, Redwood City, CA, USA) that performs a comprehensive analysis of precursor proteins and related biological processes based on the proteomic data.

Predicted changes in downstream effects

Primary data analysis indicated that some of the proteins that were differentially expressed in AF were related to cardiovascular system development and function. Possible downstream effects in the morphogenesis of cardiovascular system (p-value 4.00E-04),

Fig. 6. Analysis of the potential morphology cardiovascular system and cardiogenesis-associated downstream effects of differentially expressed peptides and their precursor proteins. Precursor proteins, diseases and functions are represented as nodes, and the biological relationships between nodes are represented as lines with arrows. All lines are supported by at least one literature reference from Ingenuity Pathway Analysis. The intensity of the node colour indicates the degree of up- (green) or down- (red) regulation.

Fig. 7. Networks based on the 183 differentially expressed precursor peptides. (A) Network A: Cardiovascular System Development and Function, Skeletal and Muscular System Development and Function, Cancer. (B) Network B: Hereditary Disorder, Neurological Disease, Connective Tissue Disorders. Colorized nodes represent input precursor proteins. Down-regulated proteins are coloured red, up-regulated proteins are coloured green.

cardiogenesis (p-value 8.06E-03), hypoplasia of myocardium (p-value 4.24E-03), and abnormal morphology of cardiovascular system (p-value, 7.60E-03) were predicted for the VSD group. The differentially expressed precursor proteins related to cardiogenesis were shown in Fig. 6.

Predicted changes in upstream regulation

Analysis of potential molecular interaction networks of the 183 differentially expressed proteins identified 11 high scoring networks. Both 'Cardiovascular System Development and Function, Skeletal and Muscular System Development and Function, Cancer' (Fig. 7A) and 'Hereditary Disorder, Neurological Disease, Connective Tissue Disorders' (Fig. 7B) networks were significantly perturbed.

Putative bioactive peptides in VSD formation

All identified peptides in cardiovascular field located within the domains of their respective precursor proteins are listed in Table 3, including the amino acid sequences, amino acids with PTM, peptide location and domain/region description. In addition, we also the listed upstream entrez gene name, location, family and gene ID for human, mouse

Table 3. Identified differentially expressed peptides within functional domains of cardiovascular-related proteins, their post-translational modifications (PTM)

Protein	Peptide	PTM	Peptide location	Domain/region description	Fold change
ATM	QNILIN		2874-2879	PI3K/PI4K	1.555
CO1A1	GPRGPPGSAGAPGKDGL NGLPGPIG	Deamidation	1139-1163	Triple-helical region	-1.636
CO2A1	GRVGPPG		903-909	Triple-helical region	-1.899
CO3A1	GPPGPQGLQGL		633-643	Triple-helical region	-1.734
CO4A1	TGRAGSPGR	Dehydration	555-563		1.938
	KGERGPLGPP	Acetylation (N-term)	582-605		-1.701
	PIGDK		613-617		2.762
	PGLPGPMGPPGLPG	Oxidation (M)	1247-1260	Triple-helical region	-1.787
	GVTGLVGIPGPPGIP	Carbamylation	1384-1398		-2.297
	GAPGQKGEMGPA		1402-1413		-1.508
	GAPGQKGEMGPAG		1402-1414		-1.7
CO5A1	PGAPLLPPLLL		15-25	signal peptide	-1.615
COAA1	KPGVPGL		107-113	Triple-helical region	-1.358
COFA1	PVRPH		960-964	Nonhelical region 6	3.474
	NGSFLMSGPPGLP	Deamidation (NQ)	1046-1058	Nonhelical region 8	1.51
COIA1	LKDEL		1654-1658	Nonhelical region 11	1.518
ERBB4	DSSSCVRA		300-307	Compositional bias, Cys-rich	2.306
NOTC4	YSGPTCH		424-430	EGF-like 10	2.832
PKD1	LGLGLWL		13-19	signal peptide	2.292
MINT	RNYQH		235-239	DNA-Binding	1.738
TITIN	MSGYPL	Amidation	1314-1319	Ig-like 5	2.122
	GPMIH	Hydroxylation	4288-4292	Ig-like 23	-2.37
	DGAALSPSPN	Dehydration	4514-4523	Ig-like 25	2.208
	ISNEVGSSSCE	Phosphorylation (STY)	5018-5028	Ig-like 30	1.846
	AVASSAVLVI	Amidation	8027-8036	Ig-like 62	-1.506
	SAELR		9751-9755	Ig-like 78	-1.562
	KTKANVT		12666-12672	Ig-like 84	1.697
	SKTVSV		18224-18229	Ig-like 105	-1.533
	RPGEENLH	Carbamidomethylation	19027-19035	Fibronectin type-III 38	-1.969
	VRGRPAKVT	Hydroxylation	19557-19566	Ig-like 109	1.701
	VSDLK		19629-19633	Fibronectin type-III 42	-1.634
	RIFVPIKGRAP		21722-21733	Ig-like 114	3.349
	VHAGETFL	Methyl ester	25646-25654	Ig-like 125	-1.814
	TLSYVVT	Acetylation (N-term)	30123-30129	Fibronectin type-III 119	1.691
	VAAVNKVG	Methyl ester	30344-30351	Fibronectin type-III 121	3.982

Table 4. Homogeneous analysis of differently expressed peptides in cardiogenesis

Symbol	Entrez Gene Name	Location	Family	Entrez GeneID Human	Entrez GeneID Mouse	Entrez GeneID Rat
ATM	ATM serine/threonine kinase	Nucleus	kinase	472	11920	300711
CASZ1	castor zinc finger 1	Nucleus	enzyme	54897	69743	313713
COL10A1	collagen type X, alpha 1	Extracellular Space	other	1300	12813	
COL15A1	collagen type XV alpha 1	Extracellular Space	other	1306	12819	
COL18A1	Collagen type XVIII alpha 1	Extracellular Space	other	80781	12822	85251
COL1A1	collagen type I alpha 1	Extracellular Space	other	1277	12842	29393
COL2A1	collagen type II alpha 1	Extracellular Space	other	1280	12824	25412
COL3A1	collagen, type III alpha 1	Extracellular Space	other	1281	12825	84032
COL4A1	collagen type IV alpha 1	Extracellular Space	other	1282	12826	290905
COL5A1	collagen type V alpha 1	Extracellular Space	other	1289	12831	85490
E2F8	E2F transcription factor 8	Nucleus	transcription regulator	79733	108961	308607
ERBB4	erb-b2 receptor tyrosine kinase 4	Plasma Membrane	kinase	2066	13869	59323
FZD5	frizzled class receptor 5	Plasma Membrane	G-protein coupled receptor	7855	14367	317674
HOXA3	homeobox A3	Nucleus	transcription regulator	3200	15400	103690130
NOTCH4	notch 4	Plasma Membrane	transcription regulator	4855	18132	406162
PKD1	polycystic kidney disease 1	Plasma Membrane	ion channel	5310	18763	24650
SPEN	spen family transcriptional repressor	Nucleus	transcription regulator	23013	56381	690911
TTN	titin	Cytoplasm	kinase	7273	22138	84015
WT1	Wilms tumor 1	Nucleus	transcription regulator	7490	22431	24883

and rat in Table 4, which also indicated that most peptides were derived from evolutionarily conserved proteins or protein domains. Titin (TTN) was represented by the highest number of peptides, many of which are part of Ig-like or Fibronectin type-III domains. Predicted changes in upstream regulation revealed that most changes associated with TTN were related to Pkc(s) and NF- κ B pathways.

Identification of candidate enzymes

We chose "VAAVNVKG" who has the highest fold change and is also in functional domain of Titin in Table 3 as our target. A search of the peptidase database (MEROPS) (30) identified a number of candidate peptidases that would likely cleave the starting and ending bond, also referring to Arg-Val and Gly-Met sites of VAAVNVKG (Table 5). This list included peptidases and proteases from different classes (serine, cysteine, and metallo), as well as soluble and membrane-bound forms of the enzyme. According to this list, the cathepsin K and matrix metalloproteinase-2 can cleave VAAVNVKG at both cleavage sites, suggesting that a single enzyme may regulate VAAVNVKG.

Discussion

Technological advances facilitating the rapid analysis of whole genomes has yielded large amounts of gene sequence and expression data that can be utilized by proteomics and peptidomics approaches. Peptidomics is an emerging branch of proteomics that targets endogenously produced protein fragments [10]. Rather than examining a sample for intact proteins, peptidomics identifies endogenous protein fragments.

Recent technological advances in proteomics and peptidomics have been actively utilized to investigate AF proteins in order to better understand this complex biological

Table 5. Peptide “VAAVNV-KG”-degrading enzymes predicted by MEROPS. Cathepsin K and matrix metalloproteinase-2 (in bold) are the only two enzymes that are predicted to cleave at both VAAVNV-KG cleavage sites. Cathepsin K and matrix metalloproteinase-2 (in bold) are the only two enzymes that are predicted to cleave at both VAAVNV-KG cleavage sites

Peptidase	Enzyme class
R-V cleavage site	
cathepsin K	Cysteine
matrix metalloproteinase-2	Metallo
matrix metalloproteinase-3	Metallo
matrix metalloproteinase-7	Metallo
ADAM17 peptidase	Metallo
ADAMTS4 peptidase	Metallo
neprilysin	Metallo
granzyme B (Homo sapiens-type)	Serine
kallikrein-related peptidase 5	Serine
kallikrein-related peptidase 12	Serine
kallikrein-related peptidase 14	Serine
granzyme A	Serine
mesotrypsin	Serine
hepatocyte growth factor activator	Serine
urokinase-type plasminogen activator	Serine
t-plasminogen activator	Serine
plasmin	Serine
prosemin	Serine
trypsin-2 type A	Serine
matriptase	Serine
matriptase-2	Serine
macrophage-stimulating protein	Non-peptidase homologue
furin	Serine
PCSK2 peptidase	Serine
PCSK6 peptidase	Serine
G-M cleavage site	
cathepsin L	Cysteine
cathepsin S	Cysteine
cathepsin K	Cysteine
cathepsin B	Cysteine
ubiquitinyl hydrolase-L3	Cysteine
ubiquitin-specific peptidase 5	Cysteine
ubiquitin-specific peptidase 4	Cysteine
ubiquitin-specific peptidase 2	Cysteine
ubiquitin-specific peptidase 7	Cysteine
usp1	Cysteine
ubiquitin-specific peptidase 9Y	Cysteine
ubiquitin-specific peptidase 18	Cysteine
autophagin-2	Cysteine
matrix metalloproteinase-2	Metallo
matrix metalloproteinase-9	Metallo
carboxypeptidase Q	Metallo

fluid and to discover disease-specific biomarkers. Putative markers including intra-amniotic infection, premature rupture of amnion, and Down syndrome have been recently discovered or reanalysed [19-21]. We studied AF from normal healthy and VSD fetuses because AF is fetal in origin and so reflects the genotypic constitution and physiological status of the developing fetus. Although the collection of the biofluid through amniocentesis may be invasive, aliquots of it may be taken from routine analysis and the detailed characterization of AF dynamics in relation to pathological occurrences may lead to the discovery of new biomarkers for better prevention approaches and early detection strategies [22]. However, AF is dynamic in nature and the composition is known to differ depending on gestational age [23]. Our data was therefore restricted to fetuses of a gestational age of 23-25 weeks, the period during which most amniocenteses are performed and hence samples are most easily obtained.

To identify naturally occurring peptides in AF in an efficient manner, a novel and comprehensive approach was developed that involved centrifugal ultrafiltration to separate

peptides based on MW. We found a 10 kDa MWCO filter capable of removing most abundant proteins from human AF without compromising peptide recovery. We used ACN to resolve peptides that become adsorbed on the surface of high MW proteins, and MS/MS assays identified nearly 700 non-redundant peptides across both groups, of which 183 were differentially expressed in the VSD group.

Proteomics is shaped by proteolysis through protein degradation and stable cleavage products were generated by restricted proteolysis [24]. It has been reported that peptidomic analyses yielded disease biomarkers representing cleavage fragments from bodily fluids [25]. As protease's specificity can be reflected by the cleavage events catalyzed by protease, different cleavage sites may represent different protease activities. We analyzed the cleavage sites of all the differentially expressed peptides (Fig. 4D and 4E). We can see the cleavage sites among the 20 amino acids were distinctly different, which is worth our further exploration on changes in protease function. By analyzing cleavage site of "VAAVNVKG" we found protease cathepsin K and matrix metalloproteinase-2 can cleave VAAVNVKG at both cleavage sites, and previous studies have reported expression of metalloproteinase-2 during early cardiac and neural crest morphogenesis [26], which needs our further exploration. Because the MEROPS database cannot account for uncharacterized peptidases or proteases, we intend to either chemically inhibit peptidase activity *in vitro* experiments or genetically knock out the given peptidase in model animal as previously reported [27] to assess the global changes in the peptidome.

There exists much literature discussing the association between these proteins and cardiac function. For example, ataxia-telangiectasia mutated (ATM) protein and Prkdc collaborate to maintain genomic stability during gastrulation and early organogenesis [28]. COL1A1, COL2A1, and COL3A1 have been associated with Ehlers-Danlos syndrome, and type IV variant of this disease with symptomatic coronary artery dissection revealed impaired secretion and abnormally slow electrophoretic migration of type III collagen (COL3A) [29]. Overexpression of pro- $\alpha 3(V)$ (COL5A) mRNA was observed in fetal heart and lung, and moderately high levels were detected in certain structures of the adult human heart [30]. COL18A1 inhibits the migration of endothelial cells *in vitro* but has no effect on the proliferation of these cells [31]. PKD1 knock-out mice embryos developed hydrops, cardiac conotruncal defects and renal cystogenesis [32]. Mice lacking ErbB4 exhibit defects in cranial neural crest cell migration and die because of the resultant defective heart development [33]. WT1 null mice suffer oedema and die at midgestation from malformed hearts [34]. MINT knock-out mice embryos had defects in cardiac septum and muscle tissue, consistent with Notch signalling-associated regulation of heart development [35]. McMahon et al. [36] proposed that COL4A1 and COL4A2 could be candidate genes for congenital heart disease (CHD) with a deletion in 13q within the 6Mb critical region for cardiac development. All this perspective has given us a reason to consider them as bioactive peptides.

Titin (TTN), also known as connectin, is a very large (3.4 MDa) protein and plays an important role movement generated by skeletal and cardiac muscle contraction [37]. Most previous studies on TTN have focussed on cardiomyopathy, and over 70 TTN mutations have been found to cause familial dilated cardiomyopathy [38-43]. During heart development, changes in Titin expression are likely to impact functional transitions and diastolic filling behaviour by regulating sarcomere compliance and sarcomere homeostasis [44, 45]. Titin participates in the formation of cardiac muscle fibers and muscle tissue morphogenesis [46]. In the present study, we found 15 differentially expressed endogenous peptides derived from TTN mapped to functional domains of this protein. Whether these peptides were derived from TTN degradation or from newly synthesized material, their differential abundance in VSD AF may reflect changes in the expression and function of TTN in VSD fetuses, and they could therefore be putative bioactive peptides for VSD.

Our results indicated that changes in TTN were mainly related to Pkc(s) and NF- κ B signalling pathways, suggesting it may be through these pathways that the differentially expressed proteins mediate malformation of the ventricular septum. Ahmad [47] observed mRNA and protein levels of titin in endomyocardial biopsy of DCM patients, and TNF- α may

modulate the expression of these proteins via NF- κ B pathway. Hidalgo [48] reported that PKC phosphorylation of titin is a novel and conserved pathway that links myocardial signaling and myocardial stiffness. Network analysis indicated that most of the differentially expressed proteins could also participate in other signalling pathways related to cardiovascular development such as the MAPK, ERK and GRB2 pathways. Biological interaction networks serve as useful tools for useful for postulation of new hypotheses, and can provide important insights into diseases such as VSD.

In summary, our study provides a profile of dysregulated peptidomic analysis in human amniotic fluid with VSD for the first time. It suggests a large amount of peptides participate in heart development and provides a background resource for future functional investigations of peptides related to VSD. Larger population study is required to improve statistical meaning. And further studies are necessary to reveal possible biological functions and molecular mechanisms exerted by these peptides in the process of VSD formation.

Abbreviations

ACN (Acetonitrile); CHD (Congenital heart disease); DCM (Dilated cardiac myopathy); GO (Gene Ontology project); IPA (Ingenuity Pathway Analysis); KDa (Kilo Dalton); LC-MS/MS (Liquid chromatogram tandem mass spectrometry); PTM (Post translational modification); VSD (Ventricular septal defect); WT1 (Wilms tumor protein); TMT (Tendam mass tag).

Acknowledgements

This work was funded by a grant from the National Natural Science Foundation of China (Grant No. 81370278 and 81570209). Dr. Fu-Qiang Wang is acknowledged for assistance with mass spectrometry.

Disclosure Statement

Authors have nothing to disclose.

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