Kininogen-1 as a protein biomarker for schizophrenia through mass spectrometry and genetic association analyses

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Background. Schizophrenia (SCZ) is a complex and severe mental illness. There is a lack of effective biomarkers for SCZ diagnosis. The aim of this study was to explore the possibility of using serum peptides for the diagnosis of SCZ as well as analyze the association of variants in genes coding for these peptides and SCZ.

Methods. After bead-based fractionation, the matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF/MS) technique was used to identify peptides that showed different expressions between 166 SCZ patients and 201 healthy controls. Differentially expressed peptides were verified in a second set of sample (81SCZ patients and 103 healthy controls). The association of SCZ and three tagSNPs selected in genes coding for differentially expressed peptides was performed in 1,126 SCZ patients and 1,168 controls.

Results. The expression level of peptides with m/z 1945.07 was significant lower in SCZ patients than in healthy controls ($p<0.000001$). The peptide with m/z 1945.07 was confirmed to be a fragment of Kininogen-1 . In the verification tests, Kininogen-1 had a sensitivity of 95.1% and a specificity of 97.1% in SCZ prediction. Among the three tagSNPs (rs13037490, rs2983639, rs2983640) selected in the Cystatin 9 gene (*CST9*) which encodes peptides including Kininogen-1, tagSNP rs2983640 had its genotype distributions significantly different between SCZ patients and controls under different genetic models ($P<0.05$). Haplotypes CG (rs2983639-rs2983640) and TCG (rs13037490-rs2983639-rs2983640) were significantly associated with SCZ (CG: OR=1.21, 95%CI=1.02-1.44, $P=0.032$; TCG: OR=24.85, 95%CI=5.98-103.17, $P<0.0001$).

Conclusions. The present study demonstrated that SCZ patients had decreased expression of Kininogen-1 and genetic variants in Kininogen-1 coding gene *CST9* were significantly associated with SCZ. The findings from both protein and genetic association studies suggest that Kininogen-1 could be a biomarker of SCZ.

1 **Kininogen-1 as a protein biomarker for schizophrenia through mass**

2 **spectrometry and genetic association analyses**

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Abstract

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Conclusions. The present study demonstrated that SCZ patients had decrease   expression of Kininogen-1 and genetic variants in Kininogen-1 coding gene *CST9* were significantly associated with SCZ. The findings from both protein and genetic association studies suggest that Kininogen-1 could be a biomarker of SCZ.

Introduction

48   Schizophrenia (SCZ) is a complex psychotic disorder. It is among the top 25 mental illness in the

49   world (Owen et al. 2016; Rodriguez 2015). Although the prevalence of SCZ is not high (0.5%-

50   1.2%) (Huang et al. 2019) , the health and economic burden was enormous for patients and their

51   families as well as the society (Chong et al. 2014). To date, the etiology and pathogenesis of SCZ

52   are still unknown. The diagnosis of SCZ is mainly based on descriptive and observation criteria

53   to assess SCZ symptoms described by patients and their family members or observed by medical

54   workers. So far, no biochemical markers or screening tools are available for SCZ diagnosis

55   (Santa et al. 2017). Thus, it is important to identify SCZ biomarkers with high specificity and

56   sensitivity, especially those present in body fluids which are easily obtained.

57   It is well known that SCZ is a genetic disorder. Candidate gene or genome-wide association

58   studies have been applied to identify genetic factors for SCZ. Even though genes carrying SCZ-

59   associated variants have been identified (Ghazaryan et al. 2016; Yoosefee et al. 2016), the

60   mechanism by which how these genetic variants influence the vulnerability of individuals to

61   SCZ is waiting to be explored. Since protein is the implementer of gene function, proteomic

62   techniques such as mass spectrometry (MS) have been widely used in aiding in the diagnosis and

63   identifying biomarkers of diseases including SCZ (Nascimento & Martins-de-Souza 2015).

64   Mass spectrometry (MS) is an important technique for proteomics analysis. It separates and

65   determines proteins according to the difference in mass-to-charge ratios (M/E) between different

66   ions (Aebersold & Goodlett 2001). A variety of MS techniques have been used in clinical

67   application (Cho et al. 2015). For example, matrix-assisted laser desorption ionization/time-of-

68   flight mass spectrometry (MALDI-TOF/MS), a sensitive analytical technique, has been

69   increasingly used to evaluate the expression of specific proteins and peptides to detect

70   biomarkers of diseases including psychiatric disorders using biological samples from patients

71   and controls (Ding et al. 2015; Huang et al. 2017; Kelley et al. 2018; Lo et al. 2016).

72   It is common that genes, proteins, and environment as well as the interaction among them

73   lead to disease vulnerability. The exploration of disease biomarkers should conduct at multiple

74   levels (Sokolowska et al. 2015). In the post-genomic era, genomics and proteomics studies are

75 still two important areas that complement each other. Even though genetic variants may

76 influence the structure and expression of proteins, variation in genes may not necessarily result

77 in protein expression changes. However, changes in protein expression may reflect gene

78 transcriptional. Recently, Comes et al. applied the proteomics approach in the identification of

79 blood biomarkers for psychiatry disorders (Comes et al. 2018). They integrated proteomic

80 findings from the peripheral blood and the genetic loci identified from enrichment analysis based

81 on 30 published studies. With the availability of genomic data of a big sample the proteomic

82 approach was demonstrated to be an essential method in discovery of biomarkers for disease

83 including SCZ.

84   In present study, we initially used the MALDI-TOF/MS method to compare expression

85 levels of serum proteins between SCZ patients and control subjects. We then screened candidate

86 genes that code for differentially expressed proteins. Finally, we analyzed the association

87 between polymorphisms of protein-coding gene and SCZ. Our aim was to explore etiology and

88 diagnosis biomarkers of SCZ at both gene and protein levels.

89 Materials and methods

90 Ethics statement

91 Ethics approval for the study was granted by the Ethics Committee of the School of Public

92 Health, Jilin University (2014-05-01). All participants provided written informed consent.

93 Study overview

94 To investigate the cause and find specific laboratory diagnostic biomarkers of SCZ, we used the

95 bead-based fractionation method and the MALDI-TOF/MS technique to compare proteomes

96 between first onset SCZ patients and healthy controls to identify specifically expressed proteins

97 in SCZ patients. Verifications were conducted in another group of SCZ patients and healthy

98 controls. Then, we searched for genes coding for differentially expressed proteins based on the

99 National Center for Biotechnology Information (NCBI) database. Case-control genetic

100 association analysis was used to explore the association between candidate gene polymorphisms

101 and SCZ.

Subjects

Serum samples for protein analysis were collected from 247 first-episode SCZ patients and 304 healthy volunteers. These first-episode SCZ patients have never taken psychiatric medications and did not receive electroconvulsive therapy in the past 3 months. DNA samples for case-control genetic association study were extracted from the peripheral blood of 1,126 SCZ patients and 1,168 healthy controls. All patients were recruited from the Changchun Mental Hospital as well as all healthy volunteers from the First Hospital of Jilin University.

All patients participated in present study were diagnosed by two independent psychiatrists. All healthy controls had no mental diseases or history of serious illness, and had not taken medications for at least two weeks. All subjects were Chinese Han in this study.

Peptide extraction and MALDI-TOF/MS analysis

Peptides were extracted by copper-chelated magnetic beads. Five microliter of homogeneous magnetic beads solution and 50μl of binding solution were added into a 0.2-ml tube and mixed thoroughly. This tube was put in a magnetic bead separator (MBS) to fix the beads, and the supernatant was aspirated. Then 5μl of serum and 20μl of binding solution were added and mixed, and a MBS was used to fix the beads and the supernatant was aspirated again. After washing magnetic beads with 100μl of the washing solution, the beads were collected with MBS and the bound peptides were eluted using 20μl of the elution solution. For target preparation, one microliter of mixture containing one microliter of eluent and one microliter of the matrix (HCCA) solution was loaded onto a 600μm-diameter spot size 384 MTP target plate (Bruker Daltonics) and left to dry.

Analysis of the processed samples was conducted by the Ultraflex^{TM} III MALDI-TOF/MS instrument (Bruker Daltonics) equipped with an N_2 337 nm laser.

Candidate gene and SNP selection

The NCBI database was used to select the coding gene of Kininogen-1protein. Three tagSNPs (rs1303749, rs2983639, and rs2983640) in the candidate gene *CST9* were selected using the Haploview program (http://hapmap.ncbi.nlm.nih.gov/). The Assay designer 3.1 was used to

129   design the primers for genotyping these three tagSNPs. The primer sequences were as follows:

130   rs1303490: F: 5'-ACGTTGGATGAAGCAGTTCCCAGACTTAGG-3' R: 5'-

131   ACGTTGGATGTCTGGGAAAACTTCCCATTC-3'; rs2983639: F: 5'-ACG

132   TTGGATGTCCATCTGCCCCTAAGTGAG-3' R: 5'-ACGTTGGATGACTACCT

133   GACTATGGACTGC-3'; and rs2983640: F: 5'-ACGTTGGATGGTGTTCAAG

134   GCAAACTCCAC-3' R: 5'-ACGTTGGATGTCTGAAGAGGAAATGGGTGG-3'.

135   **DNA extraction and SNP genotyping**

136   DNA was extracted from the peripheral blood using the ClotBlood DNA Kit (Cwbio, Beijing),

137   and its concentration was detected by an ultraviolet spectrophotometer (Beckman, USA).

138   SNP genotyping was conducted using the Sequenom MassARRAY platform (San Diego,

139   CA, USA). The whole process included PCR amplification, SAP reaction, PCR extension and

140   desalination. The conditions of PCR was set as 94°C for 15 minutes to perform a hot-start, 94°C

141   for 20 seconds to denature, 56°C for 30 seconds to anneal, 72°C for one minute for 45 cycles to

142   extend, 72°C for three minutes to incubate. Then, the PCR product was incubated with SAP

143   (Sequenom, Inc., San Diego, CA, USA) at 37°C for 40 minutes. After extension and desalination,

144   the final products were analyzed by the MassARRAY software (Sequenom, USA).

145   **Statistical methods**

146   The protein spectrum analysis was conducted using the FlexAnalysis 3.0 software and the

147   ClinProTools 2.1 software. The support vector machine (SVM) and k-nearest neighbor (K-NN)

148   algorithms were used to perform class prediction, and after the calculation, statistical analysis

149   results were cross-validated. For gene polymorphism analysis, demographic characteristics were

150   compared using the χ^2 analysis and rank sum tests. Hardy-Weinberg equilibrium (HWE) was

151   examined by the Chi-square (χ^2) goodness-of-fit test. The allele frequency and genotype

152   distribution of SNPs were analyzed by logistic regression analysis. Inheritance model and

153   haplotype analyses were performed by the SNPStats program

154   (https://www.snpstats.net/snpstatts), and the best model was selected based on the smallest

155   akaike information criterion (AIC) (Akaike 1974). Data were analyzed using SPSS version 19.0.

156   For all analyses, p<0.05 (two-tailed) was considered statistically significant.

157

158   **Results**

159   **Comparison and identification of serum peptides**

160   **Peptide profiles of SCZ patients and healthy controls in training groups**

161   Serum peptide profiles of 166 first onset SCZ patients and 201 healthy controls were analyzed.

162   The mean age of 166 SCZ patients (67 males and 99 females) and 201 control subjects (90 males

163   and 111 females) was 33±13 and 36±13, respectively. Serum peptide patterns of SCZ patients

164   were significantly different from those of healthy controls (supplementary Figure 1).

165   **Selection and identification of SCZ-specific serum peptides**

166   Through the t-test of ClinPro Tools software TM 2.1, with a p-value <0.000001, the m/z 1945.07

167   of peptides were significantly lower in patients compared to healthy controls (**Figure 1(A)**).

168   Using the peptide with m/z 1945.07 to identify SCZ patients from healthy controls, the area

169   under the curve (AUC) of the receiver operating characteristic (ROC) was 0.879 (**Figure 1(B)**).

170   The ion of m/z 1945.07 was confirmed to have the sequence NLGHGHKHERDQGHGHQ,

171   which corresponded to Kininogen-1, with exactly the high molecular weight of kininogen. The

172   sequence map was shown in **Figure 2**

173   **Peptide profiles of SCZ patients and healthy controls in testing groups**

174   Class prediction analyses were conducted in another group of SCZ patients and healthy controls.

175   There were 81 patients (35 males and 46 females) with mean age of 32±13 and 103 healthy

176   controls (45 males and 58 females) with mean age of 35±10. The spectra analysis of the

177   validation set classified 77 of the 81 SCZ patients as SCZ positive and 100 of the 103 healthy

178   controls as SCZ negative, revealing 95.1% sensitivity and 97.1% specificity.

179   **Association between *CST9* polymorphisms and SCZ**

180   **Demographic characteristics and Hardy-Weinberg equilibrium**

181   Among 1,076 SCZ patients, there were 591 males and 476 females with mean age of 34±12.

182   Among 1,151 healthy controls, there were 566 males and 585 females with mean age of 36±10.

183 SCZ patients and healthy controls were significantly different by age (Z=-5.697, p<0.050) and

184 sex (χ^2=8.569, p<0.050). Except SNP rs2983640 in the patient group, genotype distributions of

185 the three tagSNPs (rs13037490, rs2983639, and rs2983640) all met the norm of HWE in both

186 patient and control groups (p>0.050).

187 **Allele frequency and genotype distribution analyses**

188 For SNP rs2983640, the difference in genotype distributions was significantly different between

189 patients and controls (χ^2=9.53, *p*=0.009), but the allele frequency was not statistically different.

190 For rs13037490 and rs2983639, no significant differences were found in both allele frequencies

191 and genotype distributions between patients and controls (*p*>0.05). Details were shown in **Table**

192 **1**.

193 **Inheritance modeling analysis**

194 For SNP rs2983640, under genetic model of codominant, recessive, or overdominant, the

195 difference of genotype distributions between patient and control groups was significantly

196 different (codominant: OR=1.35, 95%CI=1.03-1.78, *P*=0.0068; recessive: OR=1.45,

197 95%CI=1.12-1.88, *P*=0.0051; overdominant: OR=0.81, 95%CI=0.68-0.97, *P*=0.02). However,

198 for SNPs rs13037490 and rs2983639, under the four different genetic models, no significant

199 difference was found between patients and controls (*p*>0.050). The best genetic model for

200 rs13037490 and rs2983639 was recessive and overdominant, respectively. The best genetic

201 model for rs13037490 and rs2983639 as well as the results from the difference models for

202 rs2983640 were listed in **Table 2**.

203 **Haplotype analysis**

204 When haplotypes with frequency of more than 0.01 being considered, haplotypes CG

205 (rs2983639-rs2983640) and TCG (rs13037490-rs2983639-rs2983640) were significantly

206 associated with SCZ (CG: OR=1.21, 95%CI=1.02-1.44, *P*=0.032; TCG: OR=24.85,

207 95%CI=5.98-103.17, *P*<0.0001). No other haplotypes showed significant associations with SCZ.

208 **Discussion**

209 Even though there have been some studies reported that Kininogen-1 was significantly reduced in SCZ

210   patients, our study was conducted in northern Han Chinese population, and we explored if

211   variants in its coding gene were associated with SCZ. We confirmed that Kininogen-1 was a

212   serum protein biomarker of SCZ, and it was lowly expressed in SCZ patients. SNP rs2983640 in

213   *CST9*, one of the coding genes of Kininogen-1, was significantly associated with SCZ.

214   Additionally, the frequency of haplotypes carrying *CST9* variants was significantly different

215   between SCZ patients and healthy controls.

216   There are two types of Kininogens, low molecular weight kininogen (LMW) and high

217   molecular weight kininogen (HMW) (Lalmanach et al. 2010), in mammals. In 1999, Irina firstly

218   reported the association between SCZ and the kallikrein-kinin system (KKS). He found that

219   plasma KKS system activity was increased in SCZ patients, and kallikrein in the brain of patients

220   was significantly reduced. This finding suggested that KKS might play a role in blood-brain

221   barrier and brain tissue damage, leading to the development of schizophrenia (Shcherbakova et al.

222   1999). Kininogen-1 is an important component of KKS. Together with factor XII, kallikreins,

223   kinins, and kininases, it is involved in the regulation of multiple organs and diverse

224   pathophysiological processes such as cardiovascular, renal, nervous system, tumor, thrombosis,

225   atherosclerosis   and cell proliferation, inflammation, and apoptosis (Albert-Weissenberger et al.

226   2013; Puchades et al. 2003; Regoli & Gobeil 2015; Rhaleb et al. 2011).

227   HMW is a multifunctional glycoprotein in the plasma. It is synthesized by hepatocytes, and

228   can be degraded to bradykinin (BK) under the action of kallikrein. BK receptors are also

229   distributed in the central nervous system and play a role in diseases of the central nervous system

230   (Chen et al. 2000). BK induces the synthesis and release of other inflammatory mediators in the

231   central nervous system and the glial tissue, causing brain tissue damages or prolonged

232   disturbance of blood-brain barrier function, which in turn can lead to brain damage (Ding-Zhou

233   et al. 2002).

234   The production of kinins is influenced by the expression of kininogen. There was evidence

235   about the mediatory role of kinins in the inflammatory response associated with different

236   neurological disorders (Guevara-Lora 2012). In neurological diseases, such as Alzheimer's

237   disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS), proteins involved in kinin

238   generation or kinin receptor function were overexpressed (Khandelwal et al. 2011; Lisak et al.

239   2011).

240   Kininogen is a member of the cystatin superfamily, which consists of type 1 cystatins

241   (stefins), type 2 cystatins, and the kininogens. The results of this study indicated that compared

242   with healthy controls, HMW was significantly reduced in schizophrenia patients. The deficiency

243   of kininogen may lead the balance between proteases and protease inhibitors to be broken down

244   in SCZ patients, resulting in the increase of homocysteine (Hcy) levels in the serum. Since Hcy

245   is neurotoxic can affect the development of the central nervous system, it may be involved in the

246   pathogenesis of SCZ. Several studies have shown that Hcy is significantly higher in SCZ patients

247   than in healthy controls (Kale et al. 2010; Moustafa et al. 2014; Numata et al. 2015).

248   Kininogen is coded by the cystatin 9 gene (*CST9*), and cystatin 9 belongs to the Cystatins

249   superfamily. It is one of 14 members of type 2 cystatins, which were found in human body

250   compartments and fluids (Ochieng & Chaudhuri 2010). In this study, we explored the association

251   between three tagSNPs in *CST9* and SCZ. Since age and gender of patients and controls were not

252   well matched, we adjusted their confounding effect in the analysis. In the HWE test, SNP

253   rs2983640 in the patient group did not meet the norm of HWE. This may be due to that

254   rs2983640 is associated with SCZ. Findings from previous studies also indicate that SNPs that

255   are not in HWE may contribute to complex diseases (Mayo 2008). For rs2983640, the genotype

256   distribution was significantly different between patients and controls under the genetic model of

257   codominant, recessive, or overdominant. Haplotypes CG (rs2983639-rs2983640) and TCG

258   (rs13037490-rs2983639-rs2983640) were found to be associated with SCZ. All these results

259   imply that polymorphisms of *CST9* were associated with the risk of SCZ. Our genetic association

260   studies not only indicated the association between *CST9* variants and SCZ, but also confirmed

261   that the kininogen-1 protein could be a biomarker for the diagnosis of SCZ.

262   However, there is still follow-up work waiting to be done in the future. For example, we

263   need to conduct the study with a larger sample to ensure having sufficient statistical power.

264   Subjects in this study were recruited in the northeast of China, and they were all Han Chinese.

265   We need to conduct studies in subjects from other areas or ethnicity groups. We also need to

266   study the effect of *CST9* interaction with other genetic or environmental factors on the

267   occurrence of SCZ. Finally, candidate biomarkers should be validated in the absence of

268   interference from other diseases.

Conclusions

270   In summary, our study showed that using the MALDI-TOF/MS technique, Kininogen-1 could be

271   a biomarker to distinguish SCZ patients from healthy controls with a high sensitivity and

272   specificity. We also confirmed this at the gene level, i.e., variants in CST9, the coding gene of

273   Kininogen-1, were associated with the risk of SCZ. Both protein and genetic association studies

274   demonstrated that kininogen-1 could be a biomarker of SCZ. Definitely, our findings should be

275   validated in a larger sample and replicated in different populations.

276

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279

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357

Figure 1

Comparisonof m/z 1945.07 expression level between schizophrenia patients and healthycontrols

A: Distribution of serum peptide with m/z 1945.07 between schizophrenia (red) and healthy controls (green); **B** :Receiver operating characteristic (ROC) of m/z 1945.07 to distinguish schizophrenia patients from healthy controls;

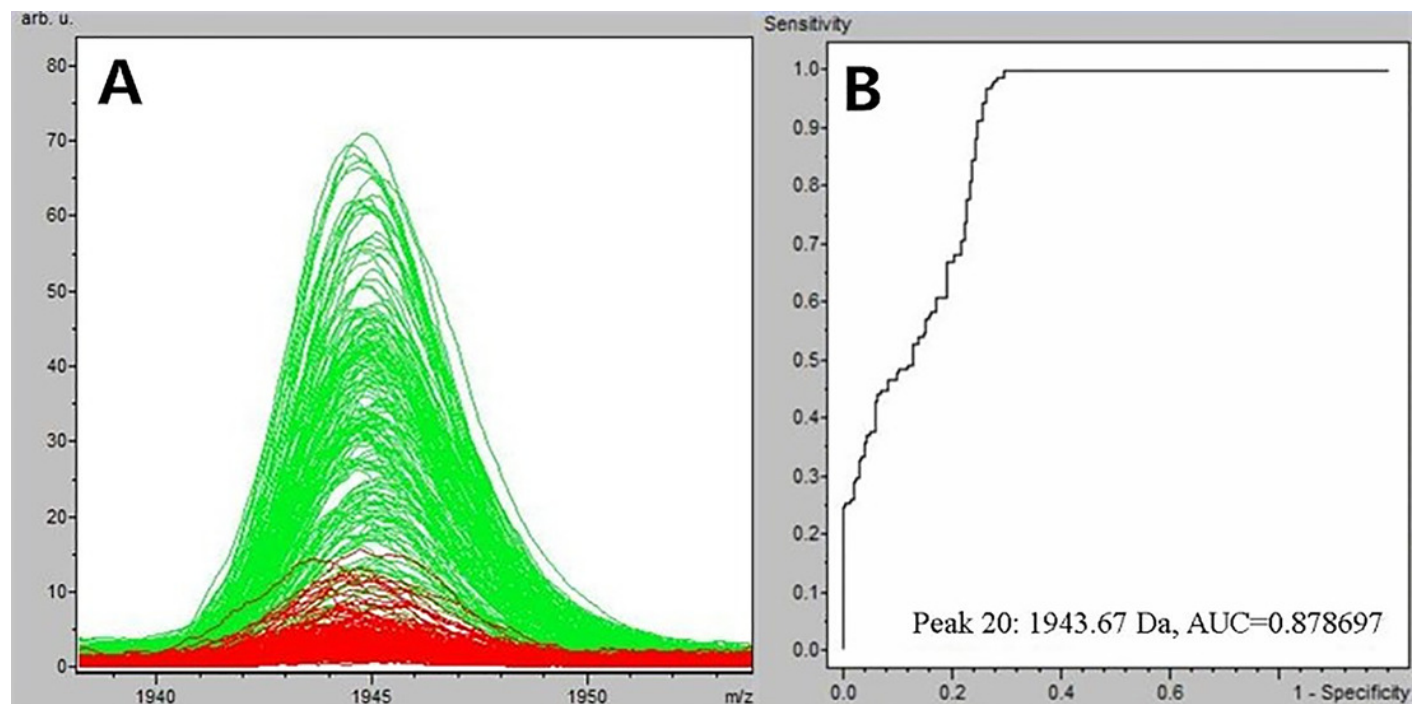
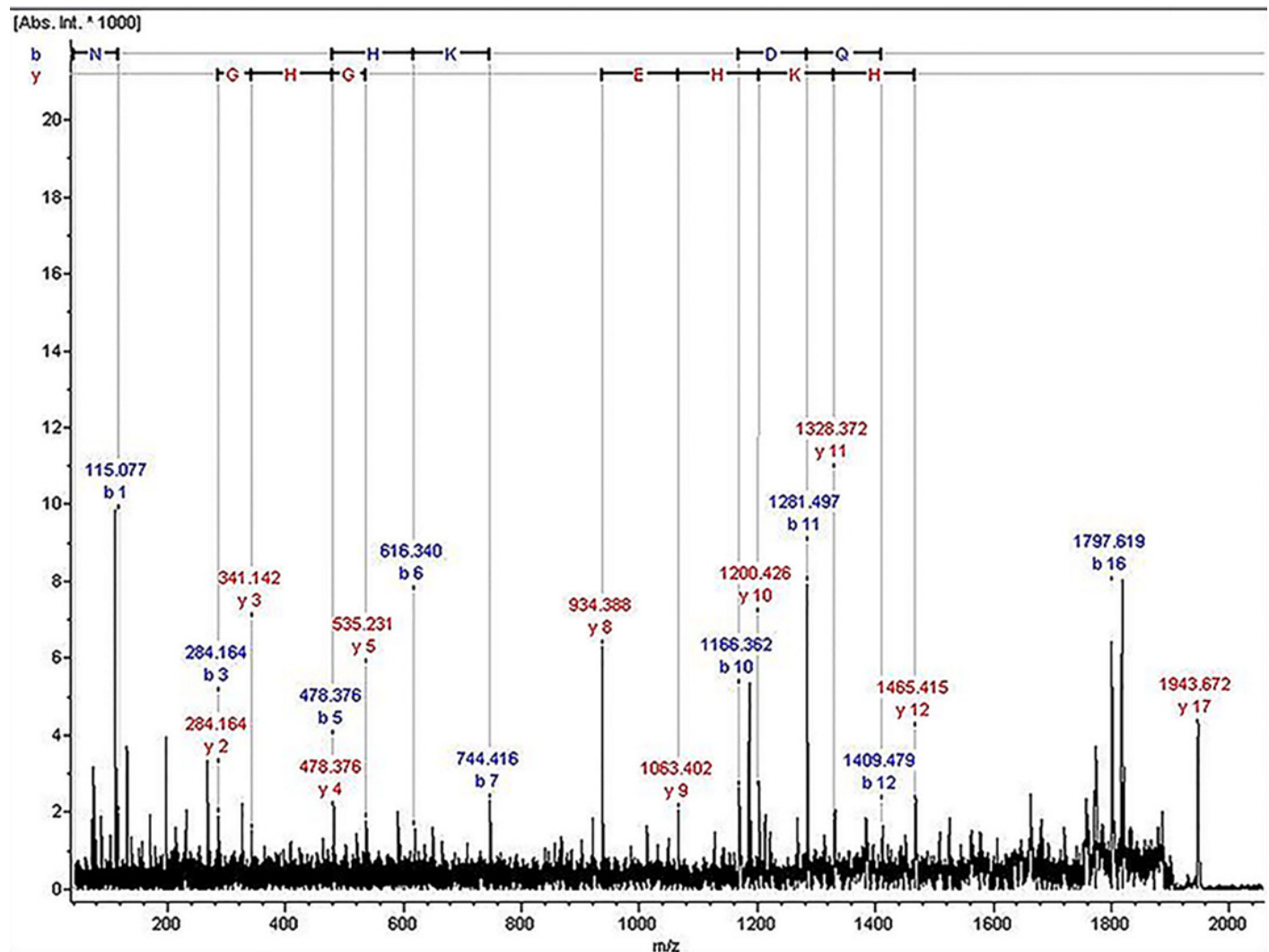



Figure 2

The sequence map of m/z1945.07

Comparison of allele frequencies and genotypedistributions of three tagSNPs between schizophrenia patients and healthy controls

Note : *P* values were adjusted for age and gender.

Table 1 **Comparison of allele frequencies and genotype distributions of three tagSNPs between schizophrenia patients and healthy controls**

SNP	Genotype/Allele	SCZ(%)	Control(%)	χ^2	P	
rs13037490	T/T	807(75)	864(76)	0.71	0.702	*Note*: P values were adjusted for age and gender.
	T/C	245(23)	264(23)			
	C/C	19(2)	16(2)			
	T	1859(87)	1992(87)	0.13	0.719	
	C	283(13)	296(13)			
rs2983639	C/C	682(65)	713(62)	4.19	0.123	
	C/T	320(30)	393(34)			
	T/T	49(5)	44(4)			
	C	1684(80)	1819(79)	0.67	0.412	
	T	418(20)	481(21)			
rs2983640	A/A	424(44)	492(43)	9.95	0.007	
	A/G	398(41)	528(46)			
	G/G	143(15)	124(11)			
	A	1246(65)	1512(66)	1.13	0.287	
	G	684(35)	776(34)			

Comparison ofinherence models between patients and controls

Note : OR (95%CI) and *P* values were adjusted for age and gender.

1 **Table 2** **Comparison of inherence models between patients and controls**

SNP	Model	Genotype	SCZ(%)	Control(%)	OR(95%CI)	*P*	AIC
rs13037490	Recessive	T/T-T/C	1024(98.2)	1128(98.6)	1.00	0.4	3026.5
		C/C	19(1.8)	16(1.4)	1.34(0.68-2.62)		
rs2983639	Overdominant	C/C-T/T	724(69.5)	757(65.8)	1.00	0.058	3005.8
		C/T	317(30.4)	393(34.2)	0.84(0.70-1.01)		
rs2983640	Codominant	A/A	419(43.8)	492(43)	1.00	0.0068	2864.5
		G/A	394(41.2)	528(46.1)	0.87(0.72-1.05)		
		G/G	143(15)	124(10.8)	1.35(1.03-1.78)		
	Recessive	A/A-G/A	813(85)	1020(89.2)	1.00	0.0051	2864.7
		G/G	143(15)	124(10.8)	1.45(1.12-1.88)		
	Overdominant	A/A-G/G	562(58.8)	616(53.9)	1.00	0.02	2867.1
		G/A	394(41.2)	528(46.1)	0.81(0.68-0.97)		

2 *Note* : OR (95%CI) and *P* values were adjusted for age and gender.