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CCR5 promoter haplotype transcription complex characterization

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Abstract

CC chemokine receptor 5 (CCR5) is a major coreceptor for cell entry of human immunodeficiency virus (HIV); its expression is highly associated with virus replication and susceptibility. Single nucleotide polymorphisms (SNPs) in the CCR5 promoter play a critical role in CCR5 transcriptional regulation. HHA and HHE represent two contrasting haplotypes of CCR5 with two base pair difference in the promoter. Identifying the transcription factors (TFs) that differentially bind to the polymorphic sites (SNPs) in CCR5 haplotypes helps elucidate HIV transmission/pathogenesis. Promoter binding and two-dimensional southwestern blot analysis are coupled with HPLC-ESI-MS/MS to purify transcription complex and identify the differential TFs binding profile, including proteins bound to one haplotype in different amounts than the other and proteins specificly bound to one haplotype. This strategy has great promise for investigating how differential TF binding to CCR5 haplotypes may impact HIV-AIDS (acquired immune deficiency syndrome) susceptibility or disease progression.

Keywords

CCR5; haplotype; SNPs; HHA; HHE; promoter trapping; two-dimensional southwestern blot; transcription factors

Why do some individuals exposed to HIV-1 resist infection? Why is there so much variation in the rate of disease progression in individuals infected with HIV-1? Several host and viral factors may be responsible for this variation. Inter-individual differences in the expression of key host molecules have been recognized as critical determinants in susceptibility to HIV-1 infection and progression to AIDS. These differences may be mediated through transcriptional, translational/post-translational and epigenetic mechanisms. Among these host factors, CC chemokine receptor 5 (CCR5) has been found to be an essential coreceptor for HIV entry into CD4+ T cells.[1] CCR5, when coexpressed with CD4, supports HIV infection by HIV-1 envelope glycoprotein (Env)-mediated cell fusion/entry. CCR5 expression on the cell surface of specific leukocyte subsets is necessary for HIV-1 infection and is associated with HIV-AIDS susceptibility, HIV-1 transmission and disease progression.[2] It has been determined that there is significant inter-individual variation of CCR5 expression on HIV target cells. What causes that variation? Several polymorphisms in the open reading frame (ORF) and promoter region of CCR5 have been found to be associated with CCR5 surface expression on specific cell types. For example, a 32-bp deletion (Δ32) in the CCR5 coding region causes loss of CCR5 expression, thus resisting

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HIV entry.[3] Polymorphisms in the noncoding promoter region of CCR5 have been shown to play roles in CCR5 promoter activity, CCR5 expression, and to be associated with inter-individual differences in HIV-AIDS susceptibility *in vivo*,[4] associated with virus replication,[5] influence virus transmission and disease progression[6] and influence antiviral therapy.[7] But the molecular mechanism of these SNPs in CCR5 expression is unclear.

We have previously used an evolutionary based strategy to organize SNPs in the cis-regulatory region and CCR5-Δ32 mutation in the ORF region of CCR5 into one of seven groups of human haplotypes from HHA to HHG (HHA, HHB, HHC, HHD, HHE, HHF and HHG).[6b, 8] Among them, HHE (303A/627C) and HHA (303G/627T) are two typical haplotypes with mutation at sites 303 and 627 in the promoter region of CCR5. HHE/HHE genotype is associated with an increased risk of acquiring HIV and faster rate of disease progression to AIDS, increased CCR5 expression on T cell surface but not monocytes and reduced cell mediated immunity, by contrast, HHA is ancestral haplotype (similar to that found in chimpanzee) and is common in subjects of African descent. This haplotype is found to be associated with lowest transcriptional activity and slower disease progression in HIV^+ African Americans, and HHC haplotype was associated with slower AIDS progression rates in European Americans. In addition, CCR5 haplotypes are associated with cell-type specific effect, resulting in altered expression in specific cell types. Previous study has shown that Oct-1 binding to 627T site on HHA haplotype although bioinformatics prediction can not address this binding site due to very similar sequences between haplotypes. So in this study, HHA and HHE which represent two contrasting phenotypes are used as a model to explore how CCR5 polymorphisms in the promoter region mediate their effects on CCR5 expression by influencing differential binding of transcription factors (TFs) to SNPs in the CCR5 promoter at the transcriptional level. This will further help to elucidate inter-individual difference and variable HIV-AIDS susceptibility.

As HHA and HHE haplotypes only have a two base pair differences, it's hard to predict TFs binding to the SNPs sites by bioinformatics analysis as we mentioned above. In order to identify the differential TFs binding to SNPs in CCR5 promoter, promoter trapping (PT) is first used to purify the transcription complex bound by HHA or HHE promoters individually. In this technique, the promoter DNA is tailed with a single-stranded $(GT)_5$ at each 3′-end. After incubating tailed promoter DNA with cellular nuclear extract, a transcription complex is formed, which can be trapped by annealing to a $(CA)_5$ single strand coupled to Sepharose beads and the bound proteins are eluted with high salt buffer.[9] The PT purified transcription complex contains not only specific transcription factors which interact with response elements on a promoter, but also contains a pre-initiation complex which is composed of general transcription factor II family members (A, B, D, E, F, H) and RNA polymerase II subunits as well as mediators. In this study, we aimed to identify the specific transcription factors bound with SNPs, to avoid the contamination of high abundant proteins like TFII family members and to increase the sensitivity of specific TFs identification, two-dimensional southwestern blots (2D-SW) were then used to compare the promoter binding profile between HHA and HHE. To perform 2D-SW, proteins are separated by two-dimensional gel electrophoresis (2-DE) and the proteins on a gel are transferred to PVDF membrane. The proteins on the blot are renatured and probed with radiolabeled promoter DNA to localize the DNA binding proteins. The protein spots which disappear in the HHA or HHE binding profiles, or protein spots showing intensity variation because of different binding affinity with promoter DNA on the 2D-SW blot will be taken as differential TFs binding to SNPs of CCR5 promoter. Next, the differentially bound proteins on 2D-SW blot were identified by on-blot trypsin digestion coupled with HPLC-ESI-MS/MS. As proteins interact with promoter DNA in 2D-SW analysis occurred at 1.5 nM concentration of DNA probe, most of the unspecific, low affinity binding proteins are removed from consideration, such as pre-initiation complex components, chromatin remodeling proteins and RNA

binding proteins. Thus, identifying the different TFs will help elucidate how the protein-DNA interaction influences differential CCR5 transcription activity and differential CCR5 expression.-

Methods

All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). Poly dI:dC, dithiothreitol (DTT), iodoacetamide (IDA), zwittergent 3–16, acetonitrile (ACN) and Tris were from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose (NC), polyvinylidene fluoride (PVDF) membranes, sodium dodecylsulfate (SDS), urea, thiourea, ampholyte, IPG strips and other gel electrophoresis reagents were from BioRad Laboratories (CA, USA). Lambda exonuclease was from New England Biolabs (Ipswich, MA, USA). Pol II (N-20) and TFIIF RAP74 (C-18) antibodies were from Santa Cruz Biotechnology (California, USA); SP1 and TBP antibodies were from Upstate Inc. (Chicago, IL, USA). Trypsin (sequencing grade) was from Promega (Madison, WI, USA).

2.1 Preparation of $(GT)_5$ tailed CCR5 promoter DNA

Tailed CCR5 haplotypes, i.e. HHE and HHA, were prepared as previously reported.[9] Simply, HHE and HHA promoter DNA was first amplified separately from pGL3-HHA or pGL3-HHE plasmids by using two pairs of primers shown below: 5′-ttcagatag attatatctg gagtg-3′ and 5′-Phos-acacacacac cagatgagctgtgcaaatc-3′, which is modified at 5′-end by phosphorylation. The other pair of primers are: 5′-cagatgagctgtgcaaatc-3′ and 5′-Phos-acacacacac ttcagatag attatatctg-3′ which is also 5′-end phosphorylated. All PCR products were purified from primers by using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The two PCR products of about 800 bp were digested individually with lambda exonuclease and one strand of each PCR product was degraded because it has 5′end phosphorylation, thus producing a single strand DNA (ssDNA) with a $(GT)_5$ tail at the 3′ end. Once two ssDNAs with $(GT)_5$ tails, representing an upper and lower strand, were annealed, the tailed duplex can further be purified by $(CA)_5$ affinity chromatography because only $(GT)_5$ tailed HHA or HHE will bind $(CA)_5$-Sepharose beads. To perform this, two PCR products after lambda exonuclease digestion were combined in equal amounts and annealed (1 ml), then diluted into 10 ml TE0.1 buffer (10 mM Tris, pH7.5, 1 mM EDTA, 0.1 M NaCl) for affinity chromatography using a 1 ml bed volume $(CA)_5$-Sepharose column at 4°C. The tailed DNA bound on the column was eluted with TE buffer (10 mM Tris, pH7.5, 1 mM EDTA) containing 0.1% Tween-20 at 50°C. After DNA concentration was determined, DNA was stored at −20°C for later use.

2.2 Preparation of nuclear extract

Jurkat cells were cultured in RPMI1640 containing 10% fetal bovine serum as described previously (Mummidi 2007). Jurkat cells were stimulated with PMA (25ng/ml) and Ionomycin (500 ng/ml) for 30 minutes and then was used. PMBC were harvested from buffy coats of normal donors and were stimulated by anti-CD3/CD28 antibodies as described previously.[10] Nuclear extracts were prepared as described.[11]

2.3 Promoter trapping chromatography

Promoter trapping was performed as previously described with little modification.[9] 500 μg (100μl) nuclear extract was mixed with $(GT)_5$ tailed HHA or HHE promoter DNA (final 50 nM) in 500 μl incubation buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Tween-20) containing poly dI:dC (10 ng/μl). The mixture was incubated at 4°C to form the transcription complex, then the mixture passed over a 100 μl $(CA)_5$-Sepharose column (36 nmol DNA/ml beads) to allow the protein-DNA complex to be trapped on the column. The column was washed with 20 column volumes of incubation

buffer, and proteins bound on the column were eluted with TE0.4 buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.4 M NaCl). Eluate was desalted and concentrated using an Amicon concentrator (10 kDa cut off).

2.4 Western blotting analysis

Gels were electroblotted onto 0.2 μm pore PVDF membranes (BioRad, Hercules, CA, USA). The dilution of primary antibody is as follows: 1:500 RAP74 (C-18) and 1:2000 RNA polymerase II and TBP antibodies. Immunoreactive proteins are visualized by using 1:10,000 diluted goat anti rabbit or by using 1:5,000 diluted goat anti mouse secondary antibody-HRP conjugate (SouthernBiotech, Birmingham, Alabama, USA) as appropriate and detected by chemiluminescence (SuperSignal @ West Dura Extended Duration Substrate, Thermo Fisher Scientific, Rockford, IL).

2.5 Two-dimensional southwestern blot (2D-SW) analysis to locate DNA binding proteins

2D-SW was performed as previously reported with little modification.[12] Briefly, nuclear extract (150 μg) was mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.8% Ampholytes, 1% Zwittergent 3–10, 0.01% bromophenol blue) for two-dimensional gel electrophoresis (2-DE). The first dimension was performed using ReadyStrip IPG strips (pH 3–10, linear, 7 cm) in a PROTEAN IEF cell (BioRad), according to the manufacturer's protocol. IEF was separated at 40, 000 v·hr and 20°C. Then, the strips were equilibrated in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol, 0.0001% bromophenol blue) containing 2% DTT at room temperature for 15 min, in equilibration buffer containing 2.5% iodoacetamide for 15 min. The strips were transferred to a 12% SDS-PAGE gel for second dimensional electrophoresis using the PROTEAN II xi 2-D (BioRad) cell. After electrophoresis, the gel was transferred to PVDF membrane for southwestern blotting (SW) analysis. Proteins on 2-DE blot were blocked and renatured in incubation buffer (used for promoter trapping) containing 2.5% non-fat milk at 4°C overnight. After washing the blot thrice with incubation buffer, radiolabeled promoter DNA without a tail (final concentration was 1.5 nM) was added into incubation buffer containing 0.25% BSA and 10 ng/μl poly dI:dC and probed at 4°C overnight. The washed blot was exposed for autoradiography.

2.6 Protein identification by in-gel or on-blot trypsin digestion and HPLC-nanoESI-MS/MS

The promoter trapping eluate after desalting and concentration was separated by 12% SDS-PAGE, stained by 0.25% Coomassie Brillant Blue R-250. The gel slice was cut into ten equal pieces horizontally and digested in gel with trypsin as previously described.[13] Briefly, the gel pieces were cut into 1 mm × 1 mm small cubes and destained thrice for 30 minutes with 25 mM NH_4HCO_3/50% ACN, reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 56°C for 1 h and alkylated in the dark with 50 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 1 h. Then the gel plugs were dehydrated with 100% ACN, lyophilized by speed vacuum and immersed in 15–20 μL of trypsin (25 ng/μl) in 25 mM NH_4HCO_3 for digestion at 37°C overnight. Protein spots on 2D-SW blot were digested as previously described with little modification.[14] Briefly, the spots were cut from PVDF membrane, wetted with methanol, washed ten times with water, reduced with 10 mM DTT in 100 mM NH_4HCO_3 at room temperature for 1 hour and alkylated in the dark with 50 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 1 hour. The proteins on the blot were digested with 25 ng/μl trypsin in 25 mM NH_4HCO_3 containing 30% ACN and 0.5% Zwittergent 3–16 at 37°C overnight. After digestion, peptides were extracted twice with 5%TFA/50%ACN (50 μl). After lyophilization by speed-vacuum, peptides were resuspended into 20 μl of 0.1% TFA and analyzed by capillary HPLC-nano electrospray ionization-tandem mass spectrometry (HPLC-nanoESI-MS/MS) using a Thermo Finnigan linear ion trap mass spectrometer equipped with a nano-ESI source (LTQ-XLS;

ThermoFisher, San Jose, CA). On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC reverse phase HPLC. The column was a PicoFrit (New Objective; 50 μm i.d.) column packed to 8 cm with C18 adsorbent (Vydac 218MS, 5 μm, 300 A). The capillary LC gradient was 2–98% of 0.1% formic acid/acetonitrile over 60 min at a flow rate of 300 nL/min. MS conditions were a 2.5 kV ESI voltage, and in data dependent mode the seven most intense ions in the survey scan with an isolation window for MS/MS of 2 were fragmented at 35% relative collision energy and collision-induced dissociation (CID). MS/MS spectra were searched against the human SWISSPROT non-redundant database combined with an in-house transcription factor database (2944 sequences; 1,671,525 residues) based on probability (greater than 95%) and error-tolerant. Precursor ion mass tolerance was set to 1000 ppm and 0.8 Da for product ion mass tolerance, maximum 2 missed cleavages, carbamidomethyl cysteines and oxidized methionines as variable modifications, and an ion score threshold of 20. The proteins with two unique peptide matched were considered as high confidence, sometimes one peptide with high ion score are also considered.

Results

HHA and HHE are contrasting haplotypes with two base pair different (SNPs) in promoter region of CCR5 gene. Identifying the differential binding proteins to HHA or HHE will help elucidate transcriptional regulation of CCR5 expression. Two approaches were used to identify the proteins which bind differently to HHA and HHE: one is to combine PT, SDS-PAGE separation, in-gel digestion and HPLC-ESI-MS/MS; another is to couple 2D-SW, on-blot digestion and HPLC-ESI-MS/MS.

To separate the differential binding proteins, promoter trapping was performed individually to isolate the transcription complexes formed by HHA and HHE promoter DNA and the components in each transcriptional complex were compared. PT purification requires a tailed promoter DNA, i.e. a single strand $(GT)_5$ tail added to the duplex promoter DNA at its 3′ ends. To achieve this, one of paired primers was designed to have an $(AC)_5$ sequence and a phosphate at 5′ end, which allows that strand to be degraded by Lambda Exonuclease, leaving a single strand with the complement $(GT)_5$ tail at 3′-end. The other complementary single strand with a $(GT)_5$ tail at 3′-end was synthesized similarly, and the two single strands annealed to give the final product. We took HHE promoter as an example, as shown in Figure 1, two PCR products about 800 bp long were amplified by using two primer sets separately (Fig. 1A); one strand of PCR product, containing a 5′-end phosphate was digested by Lambda Exonuclease which resulted in a single strand with $(GT)_5$ tail about 400 nucleotides long, indicated by a star in Fig. 1B, it can be seen that a small amount of double stranded DNA remained after Lambda Exonuclease digestion presumably because addition of the 5′-end phosphate during synthesis is incomplete. To verify these are single strands (ssDNA) coming from HHE promoter DNA, they were digested by restriction endonuclease BamH1. Fig. 1C shows that the two single strands cannot be digested by BamH1, but after they were annealed, the annealed double strand was digested into two fragments which are the same as the positive control, indicating two $(GT)_5$ tailed ssDNAs annealed to form tailed HHE promoter DNA. In order to remove promoter DNA without a $(GT)_5$ tail, the annealed double strand was passed over a $(CA)_5$-Sepharose column since only $(GT)_5$ tailed promoter DNA is retained on this column by $(GT)_5$-$(CA)_5$ annealing. The transcriptional complex trapped by PT contains a pre-initiation complex capable of RNA synthesis and specific transcription factors as well as cofactors.[9] Western blot analysis shows that preinitiation complex components TATA box binding protein (TBP), RNA polymerase II (pol II) and TF IIF subunit RAP74 were trapped by CCR5 promoter trapping, shown in Figure 2. Compared with cell nuclear extract, after PT, most of proteins were removed, retaining the DNA binding proteins bound with CCR5 haplotypes, as shown in Figure 3. HHA and HHE PT

complexs show similar 2-DE protein profile, with some protein spots that differ in intensity, implicating the differential binding proteins bound to CCR5 haplotypes.

To localize the differential binding proteins, two-dimensional southwestern blot analysis (2D-SW) was performed. The proteins on a two-dimensional gel (2-DE) were transferred to PVDF membrane and renatured, then probed with radiolabeled promoter (HHA or HHE) DNA, the proteins interacting with DNA are localized by autoradiography. Comparing the 2D-SW profiles between HHA and HHE haplotypes, we determined the differential binding proteins to SNPs. The same amount of nuclear extract from Jurkat, stimulated Jurkat or peripheral mononuclear cells (PBMC) were analyzed by 2D-SW probed with radiolabeled HHA or HHE haplotype DNA separately, the DNA binding profile is shown in Figure 4. The HHE binding profiles are similar between stimulated Jurkat and PBMC, but several HHE binding protein spots are diminished in Jurkat nuclear extract. Similarly, less HHA binding protein spots were detected in Jurkat nuclear extract compared with stimulated Jurkat and PBMC. So in this study, PBMC nuclear extract was used in later experiments for PT combined with MS identification. In addition, comparing the DNA binding profiles between HHA and HHE in the same nuclear extract, it is obvious there are several differential binding proteins on 2D-SW in Jurkat, stimulated Jurkat or PBMC nuclear extract, indicating the different TFs binding to the two haplotypes or the same proteins binding there with different affinity.

To identify these differential binding proteins, the same amount of PBMC nuclear extract was used for HHA and HHE promoter trapping and the same amount of PT eluate was separated by SDS-PAGE, shown in Figure 5. The Coomassie Brilliant Blue stained gel was cut into ten equal slices for in-gel digestion by trypsin, the produced peptides were analyzed by C18 reverse phase capillary HPLC-nanoESI-MS/MS. MS data was searched against both SWISS-PROT public database and our local Transcription Factor database, and the proteins identified by Transcription Factor database search are shown in Table 1. The candidate proteins are classified into three groups: 1) HHA and HHE binding proteins; Most of them are high abundant DNA and RNA binding proteins. 2) HHA haplotype binding proteins; 3) HHE haplotype binding proteins. Proteins in group 2 and group 3 may be differential TFs binding to SNPs of CCR5 promoter. At same time, the protein spots which interacted with radiolabeled promoter DNA on 2D-SW blot of PBMC were cut out for on-blot trypsin digestion and HPLC-ESI-MS/MS identification. Some spots, indicated with circles in Figure 4, have been successfully identified, as shown in Table 2. On 2D-SW profile, protein spot 2 interacted with HHA and spot 3 interacted with HHE DNA are located at the same position, database search indicates them to be interferon regulatory factor 7 (IRF7) and interferon regulatory factor 5 (IRF5), protein spot 1 probed with HHA DNA to be protein Jumonji with three peptides matched and protein spot 4 probed with HHE DNA to be cAMP-responsive element modulator (CREM).

Discussion

CCR5 expression levels vary in individuals, which is associated with different HIV-AIDS susceptibility. In this study, we explore the mechanism by which CCR5 expression is regulated by differential TFs binding to SNPs in CCR5 promoter. Previous studies have shown the SNPs sites in CCR5 promoter region or coding region affect CCR5 expression, but the transcriptional mechanism was not elucidated. We hypothesize that differential binding of TFs to the SNPs site regulate CCR5 promoter activity and expression correspondingly. In this manuscript, we use promoter trapping, two-dimensional southwestern blot coupled with HPLC-nanoESI-MS/MS to analyze the differential binding of proteins to two CCR5 haplotypes, HHA and HHE, which are two typical contrasting haplotypes causing significantly different CCR5 promoter activity and CCR5 expression. It

was found that DNA binding profile is different between HHA and HHE by 2D-SW analysis. MS analysis has identified some proteins unique to HHA or HHE haplotype transcription complexes, as well as many proteins in common. Further investigations need to quantitatively determine the specific TFs differentially binding to HHA and HHE and to clarify how the differential binding regulates CCR5 expression, which may help with the design of drugs against the differential TFs to regulate HIV susceptibility and disease prevention.

CCR5 expression on the cell surface depends on the cell type or cell lines. It has been reported that memory/activated T cells and cells of monocyte/macrophage lineage are the primary in vivo targets for HIV-1 and have high CCR5 expression levels.[15] So in this study, we compared CCR5 promoter DNA binding profiles between the Jurkat cell line and PBMC, 2D-SW analysis indicates that stimulated Jurkat cells and PBMC have similar binding profile, different from unstimulated Jurkat cells. Using PBMC nuclear extract, we have identified several differential TFs binding to HHA or HHE from promoter trapping eluates or 2D-SW blot, confirming our hypothesis.

In this study, promoter trapping was performed to purify the transcription complex formed by HHA or HHE haplotypes. Western blot analysis showed the presence of expected pre-initiation complex proteins such as RNA polymerase II (pol II), TATA box binding protein (TBP) and TFIIF subunit RAP74, which were all detected after promoter trapping. HPLC-nanoESI-MS/MS analysis of the PT eluate indicates that there are still a many proteins bound with the promoter although PT removed many others, which are presumably not involved in transcription. Some abundant proteins were found to bind both HHA and HHE, i.e. SWI/SNF complex subunit, ATP-dependent chromatin remodeling proteins; some RNA binding proteins such as special AT-rich sequence binding protein, AT-rich interactive domain-containing protein 2, U4/U6.U5 tri-snRNP-associated protein 1 and cold shock domain-containing protein E1, which will confound the identification of very low abundant TFs. Although we found there are several TFs detected only in HHA or HHE binding, their matched peptides and sequence coverage are not very high compared with the proteins binding with both haplotypes. Among the identified TFs from PT eluate, five of them are detected in HHA PT and ten of them are detected in HHE PT. It should be pointed here that ESI-tandem MS analysis itself cannot provide enough information for protein quantitation, so the differential binding proteins to HHA or HHE need further verification. Currently, stable isotope labeling of amino acid in cell culture (SILAC) is a quantitative proteomics technique which can be used to identify the TFs binding to HHA and HHE with quantifiable differences. We will explore this technique in subsequent publications.

In the identified TFs, protein jumonji and interferon regulatory factor 7 (IRF7) were found to interact with HHA haplotype, while interferon regulatory factor 5 and cAMP-responsive element modulator (CREM) bind the HHE haplotype. The two protein spots at almost the same position on 2D-SW blots of HHA and HHE were determined to be interferon regulatory factor family members: IRF7 and IRF5 individually, indicating the specificity of 2D-SW analysis since IRF7 and IRF5 have 30% identity in amino acid sequence. They are identified by 2D-SW coupled with HPLC-ESI-MS/MS, but not by PT in this study, implying that 2D-SW has the ability to clarify some of the very low abundant TFs. Protein jumonji is identified in both HHA PT and HHA 2D-SW analysis, implying the concordance the two approaches. Currently, the other protein spots on 2D-SW blots are in the process of identification by on-blot digestion and ESI-MS/MS.

IRF family members play role in induction of interferon (IFN), IFN-stimulated genes, and other proinflammatory cytokines at the transcription level. IRF-7 is essential for the induction of IFN-alpha/beta genes by the retroviruses.[16] IFN-alpha has been reported to

significantly increase of CCR5 expression on PBMC in both HIV-infected individuals and controls.[17] Recent studies from IRF5 knockout mice have confirmed a critical role for IRF5 in virus-induced type I IFN expression and proinflammatory cytokines IL-6, IL-12, and TNF-α through both histone deacetylases (HDACs) and histone acetyltransferases (HATs) association with IRF5.[18]

Among the identified TFs, cAMP response element-binding protein 1(CREB) is detected in PT of HHA while CREB3 is detected in HHE PT, and CREM is found to be interacted with HHE haplotype by 2D-SW analysis. This study shows that CREB family members are involved in CCR5 promoter binding. The CREB family members CREB-1, activating transcriptionfactor 1 (ATF-1) and cAMP-responsive element modulator (CREM) all bind to CRE sequences as homodimers or heterodimers.[19] Previous studies have found CREB to be bound with the ISREs and CRE sites in the CCR5 promoter region and CREB-1 is important inducer of CCR5 promoter activity and involved in transcriptional regulation of CCR5 expression.[20]

In conclusion, we have combined PT, 2D-SW with HPLC-ESI-MS/MS to characterize and identify the differential TFs binding with CCR5 haplotypes, i.e. YB2, CREB, jumonji, Elf-1 and recombing binding protein suppressor of hairless are found to bind HHA haplotype, while zinc finger E-box-binding homeobox 2, CDP, DNA binding protein RFX5, vacuolar protein sorting-associated protein 72, etc. are found to bind HHE haplotype, these findings verify the hypothesis that CCR5 variation in expression is regulated at transcription level by specific TFs binding to SNPs in CCR5 promoter. These identified TF candidates may play role in regulating CCR5 promoter activity and CCR5 expression by binding directly to their DNA sites on CCR5 promoter or by protein-protein interaction with TF bound with CCR5 promoter DNA. Their function in regulating CCR5 promoter activity and CCR5 expression can be explored by knock-in or knock-out strategy. But more investigations are needed to clarify the molecular mechanism of identified TFs in CCR5 inter-individual expression difference and susceptibility.

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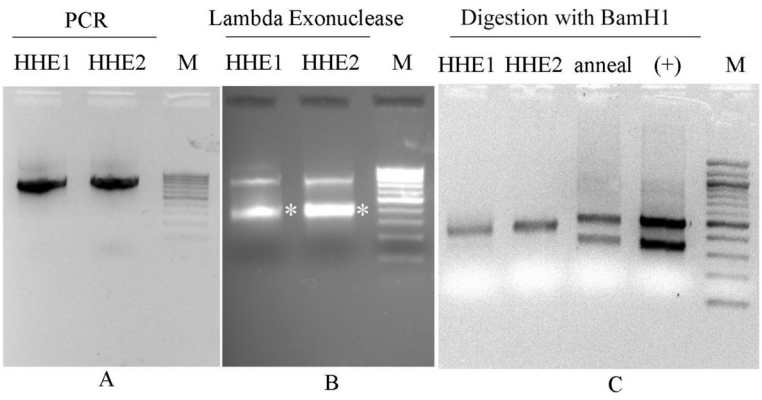

Figure 1. Preparation of $(GT)_5$ tailed HHE promoter DNA

Panel A, HHE promoter was amplified by PCR using two pairs of primers, one of which is 5′ end modified with a phosphate, two PCR product were produced, i.e. HHE1 and HHE2 and both are approximately 800 bp.. **B**, After purification with a PCR clean-up spin column, HHE1 and HHE2 were digested by lambda exonuclease (to digest the unneeded phosphorylated strand) to produce single strands containing a $(GT)_5$ tail at the 3′ end. The ssDNAs were approximately 400 nt., smaller than double stranded HHE (800 bp) and can no longer be digested at BamH1 restriction sites located in HHE promoter. The two tailed single strands were then annealed to form tailed HHE promoter, which was further purified by DNA affinity chromatography by passing over a $(CA)_5$-Sepharose column, since only tailed HHE promoter DNA will bind the (CA)5-Sepharose beads, here digested with BamH1 to show that now two fragments of approximately 500 and 300 bp are produced, clearly different in size from the single strands.. "+" shows the HHE1 double strand from PCR also digested with BamH1 give the same restriction products.

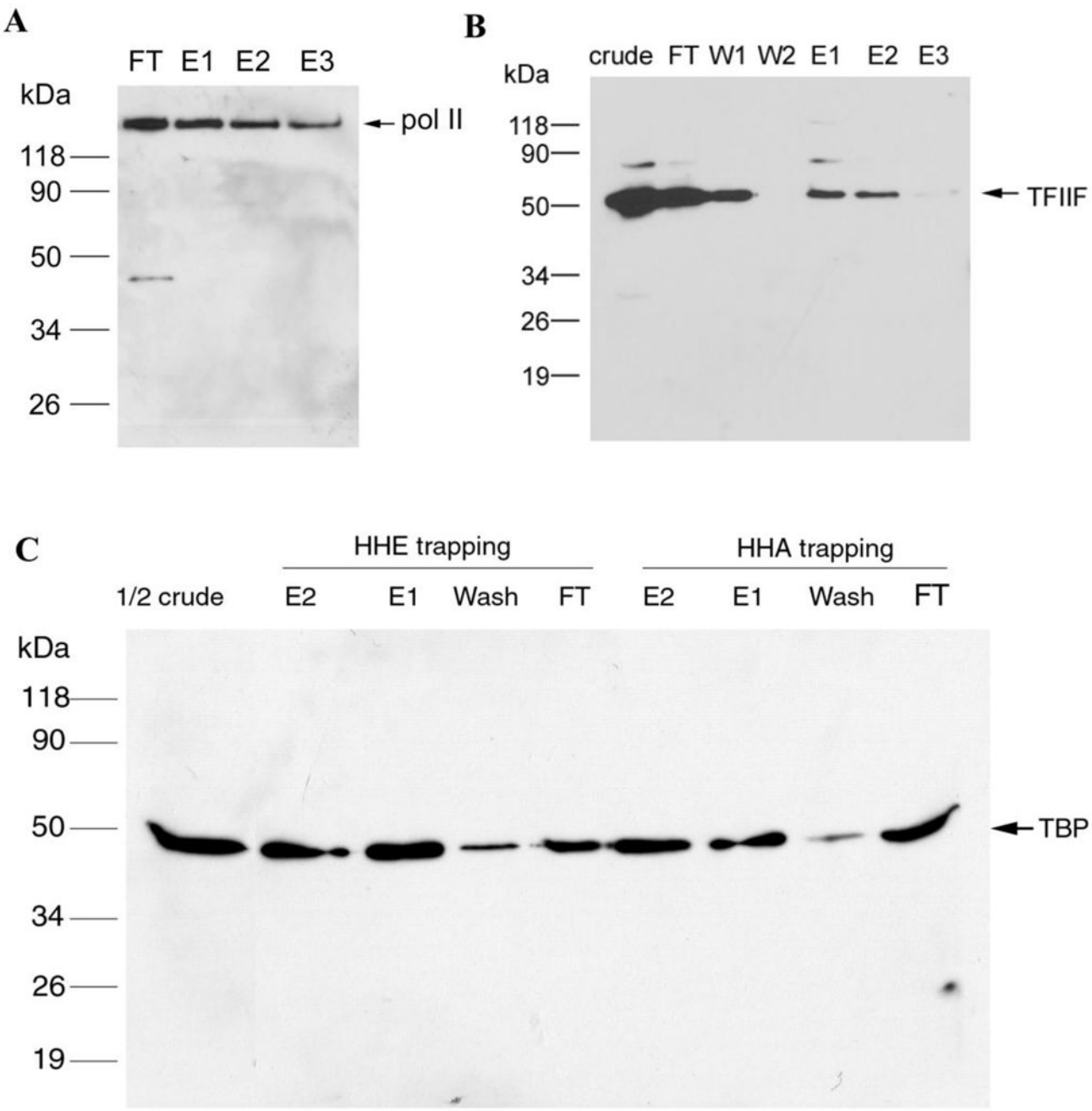

Figure 2. Western blot analysis of HHA and HHE trapping of Jurkat nuclear extract

Jurkat nuclear extract was incubated with HHA and HHE promoter DNA containing $(GT)_5$ tails to form transcription complexes and then the complex was trapped on an $(AC)_5$ column by promoter trapping (PT). The HHE PT fractions were analyzed by WB against RNA pol II (A), TFIIF RAP74 (B) and TBP (C) was compared between HHA and HHE trapping. FT, flow through; W, wash fractions of trapping, i.e. W1 and W2; E1–3, elute fraction 1 to 3; ½ crude, half as much of the initial Jurkat nuclear extract. The molecular weight standards are shown on the left.

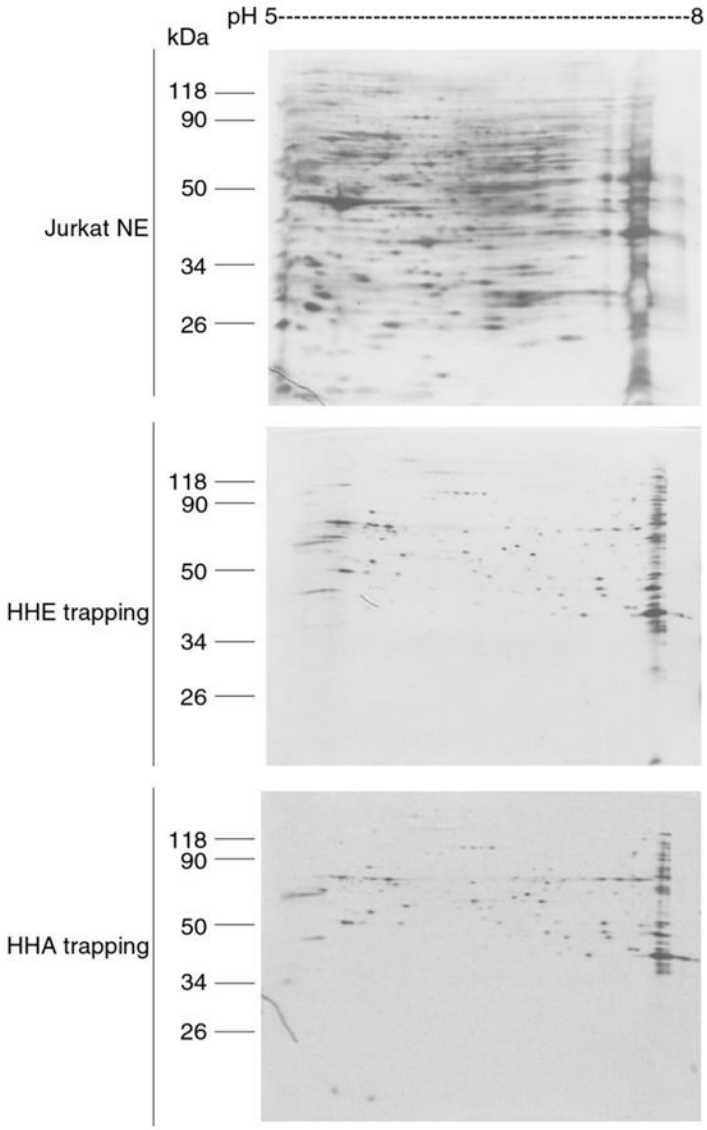

Figure 3. Silver stain analysis of two-dimensional electrophoresis gel. HHA and HHE promoter trapping

Eluate of Jurkat nuclear extract was separated by two-dimensional electrophoresis. The first-dimensional IEF was performed using an IPG strip with pH 5–8 and second dimensional electrophoresis was performed by 12% SDS-PAGE. The molecular weight standards are shown on the left.

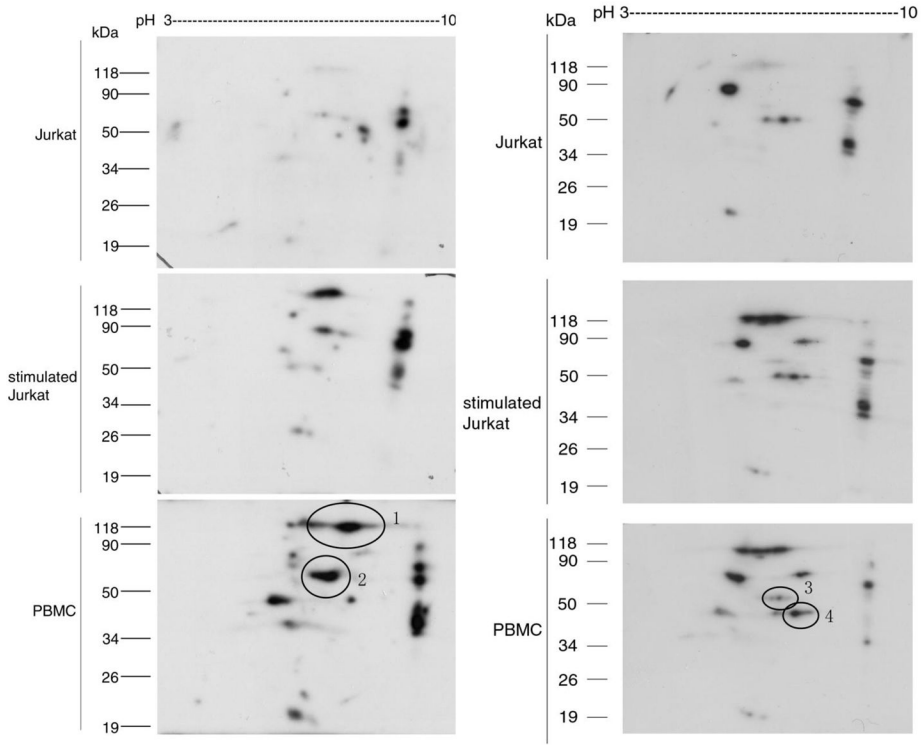

Figure 4. Two-dimensional southwestern blot analysis (2DE-SW) of Jurkat, stimulated Jurkat and PBMC nuclear extract

The nuclear extract (100 μg) was separated by first-dimensional IEF using IPG strip with pH 3–10 and second dimensional 12% SDS-PAGE electrophoresis. The proteins on the 2-DE gel were transferred to PVDF membrane and renatured in 2.5% non-fat milk in incubation buffer at 4°C overnight, then probed with radiolabeled HHA (left panels) or HHE (right panels) promoter DNA (1.5 nM) and autoradiography. The molecular weight standards is shown on the left. The protein spots circled were further identified by on-blot digestion coupled with HPLC-nanoESI-MS/MS analysis in Table 2.

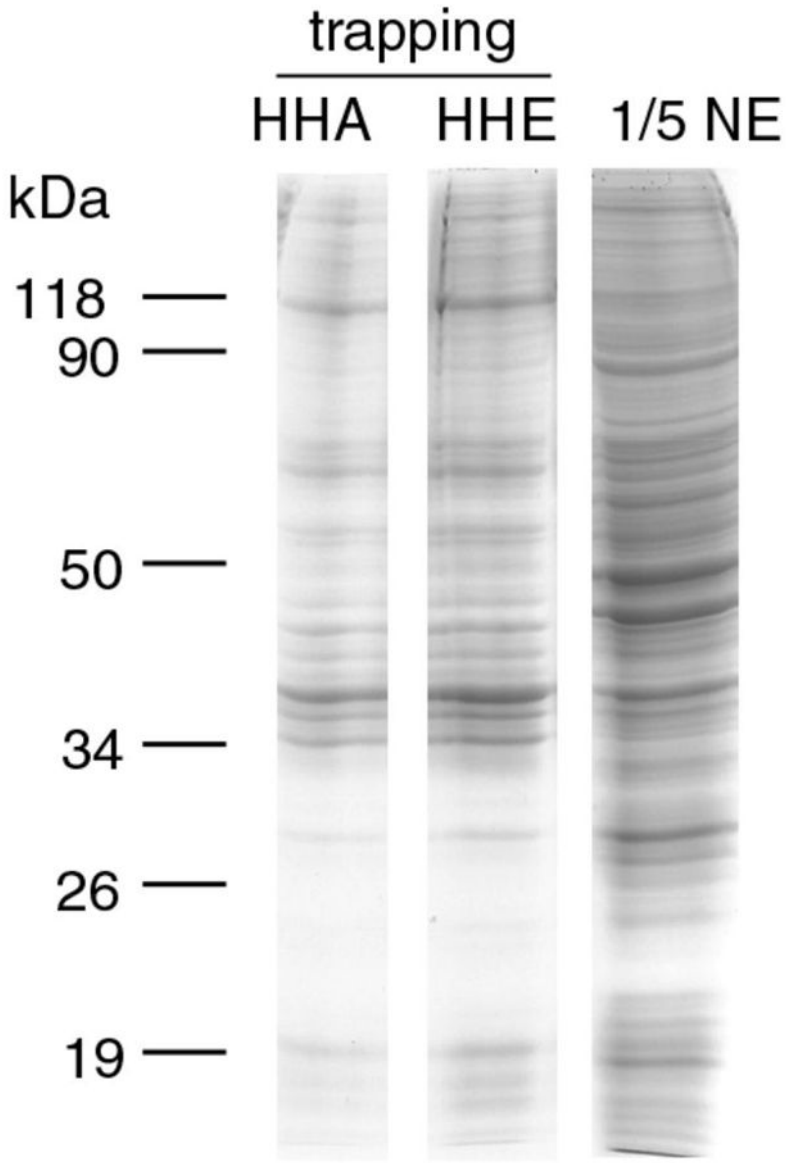

Figure 5. Coomassie stain of PBMC promoter trapping eluate

PBMC nuclear extract was incubated with 50 nM of tailed HHA or HHE promoter DNA for PT. TE0.4 buffer eluate was separated by 12% SDS-PAGE gel and stained by Coomassie Brilliant Blue. NE, PBMC nuclear extract; HHA, HHA trapping eluate; HHE, HHE trapping eluate. The molecular weight standards are shown on the left.

Table 1

Protein candidates identified by PT coupled with HPLC-ESI-MS/MS

Protein name, M.W, pI	Score, peptides matched, sequence coverage	
	HHA	HHE
SWI/SNF complex subunit SMARCC1, 122790, 5.51	1118, 20, 26%	556, 9,13%
SWI/SNF complex 170kDa subunit, 132797, 5.49	996, 21, 11%	511, 8, 9%
ATP-dependent chromatin remodeling protein, 178592, 6.20	524, 11, 11%	235, 5, 5%
special AT-rich sequence binding protein, 89271, 6.17	510, 9, 20%	253, 5, 11%
AT-rich interactive domain-containing proein 2, 197268, 7.08	189, 3, 3%	55, 1, 1%
U4/U6.U5 tri-snRNP-associated protein 1, 90200, 5.89	242, 3, 6%	37,1, 1%
cold shock domain-containing protein E1,85693, 5.84	716, 12, 21%	370,6, 15%
zinc finger protein ubi-d4, 44127, 5.94	320, 5, 25%	333, 5, 24%
cell division cycle-5 like protein, 92194, 8.22	216, 5, 10%	198, 3, 7%
metastasis-associated protein MTA2, 74976, 9.7	847, 17, 30%	760, 15, 32%
metastasis-associated protein MTA3, 66611, 8.97	146, 3, 4%	154, 4, 5%
transcriptional repressor p66-alpha, 52481, 10.46	127, 3, 9%	292, 5, 11%
transcriptionl repressor p66-beta, 65220, 9.73	101, 2, 6%	185, 3, 10%
methyl-CpG-binding domain protein 3, 32823, 5.22	55, 1, 4%	41, 1, 3%
LIM homeobox 8, 38146, 8.55	204, 32%	175, 24%
max-like protein X gamma, 33280, 8.18	61, 1, 4%	86, 2, 8%
myocyte-specific enhancer factor 2D, 55903, 7.74	207, 4, 9%	125, 1, 8%
forkhead box protein K2 or K1, 64216, 9.64 or 75411, 9.41	54, 1, 2%	100, 2, 6%
zinc finger protein, subfamily 1A(ikaros), 48258, 6.22	56, 1, 2%	228, 4, 8%
nuclease sensitive element binding protein 1(YB1), 34008, 7.00	38, 1, 7%	209, 4, 22%
Y-box-binding protein 2 (YB2), 38495, 10.80	119, 2, 7%	
CREB, 25429, 5.80	71, 1, 6%	
jumonji, AT rich interactive domain 1C, 175267, 5.40	71, 2, 2%	
ETS-related transcription factor Elf-1, 67456, 5.09	55, 1, 2%	
recombing binding protein suppressor of hairless, 54111, 8.27	79, 1, 5%	
zinc finger E-box-binding homeobox 2 isoform 1, 136361, 5.90		63, 2, 1%
CCAAT displacement protein, CDP, 159299, 5.60		56, 1, 1%
DNA binding protein RFX5, 65283, 9.35		70, 2, 4%
vacuolar protein sorting-associated protein 72, 40570, 6.09		75, 2, 5%
SREBP-1, 121599, 8.43		33, 1, 1%
CREB3, 43405, 5.28		30, 1, 1%
CCAAT/enhancer-binding protein gamma, 16408, 9.77		30, 1, 6%
HOXA-9A, 22778, 11.72		30, 1, 5%
retinoic acid receptor alpha (RAR), 50738, 8.21		31, 1, 3%
transcriptional repressor CTCFL,75698, 8.58		32, 1, 1%

* PT eluate of HHA and HHE was separated by 12% SDS-PAGE and stained by coomassie brilliant blue, shown in Figure 5. The proteins in gel were digested and identified by HPLC-ESI-MS/MS. The candidate proteins after transcription factor database search were compared between HHA and HHE PT in this table.

Table 2

HPLC-ESI-MS/MS analysis of proteins from 2D-SW blot

sample spot	Protein name	ID number	Mr. (kDa)	pI	Sequence coverage	Protein score/best ion score	Peptides identified
1	protein Jumonji	NP_004964	138648	9.46	2%	68/27	ALERR GGEGDDPAR LAELRQR
2	interferon regulatory factor 7	NP_004022.2	55635	6.12	3%	53/29	AWAVAR ALAPER
3	interferon regulatory factor 5	NP_001092099.1	57770	5.61	2%	20	MNQSIPVAPTPPRR
4	cAMP-responsive element modulator	NP_853549.1	32494	6.92	3%	21	EYVKCLESR

* Sample spots 1 to 4 on the 2D-SW blot shown in Figure 4 were cut from the blot, difested on-blot with trypsin, and analyzed by HPLC-ESI-MS/MS. The identified TFs are shown.