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Identification of Cancer Marker Vinculin from Caco-2 Cancer Cell By Using UPLC/MS Method

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Original Research Article

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Abstract: Vinculin is one the most common proteins that have been studied commonly in recent years. Vinculin has anti-tumor effect and loss of vinculin causes development of many cancer types. Because of this Vinculin is a marker and it shoulde be evaluated for invitro and clinical studies. LC/MS based proteomics is the main tool to analyze hundreds of proteins in very short time because it offers high sensitivity and accuracy. Today nano LC is the min tool for LC/MS proteomics researches but UPLC/MS based systems have been developed and offered more robust and easy way for proteomics. In this study we used normal flow UPLC/Q-TOF method to analyze caco-2 colon cancer cell line. One of our targets for this study was to identify Vinculin as a cancer marker. We digested proteins from caco-2 cells and analyzed with UPLC/MS. Recorded MS/MS data was used with Maxquant bioinformatic system. We identified 5 unique peptides that belonged to Vinculin. We used 20 ppm mass tolerance for amino acid sequence matching with uniprot human database. We made three raplicate to observe identification reprocubility. Also We used label free quantification method and calculated vinculin intensity.
Keywords: Vincul, UPLC/MS, Cancer, Proteomics, Maxquant.

INTRODUCTION

Vinculin is a ubiquitously expressed, actin-binding protein that localizes to the cytoplasmic face of integrin-mediated cell-extracellular matrix junctions (focal adhesions) and cadherin-mediated cell-cell junctions.

Normally, vinculin plays a key role in focal adhesion formation, cell proliferation and regulation of the actin cytoskeleton [1, 2]. It can be seen interactions of Vinculin with other proteins in cell in Figure 1. Especially Vinculin regulates cell surface E-cadherin expression.

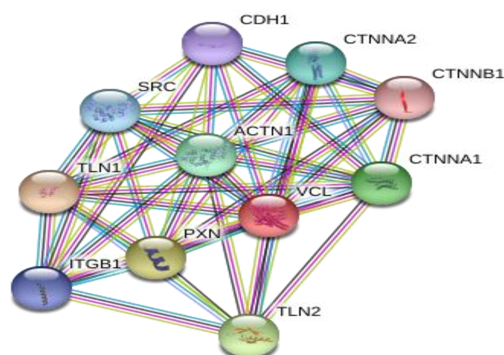

Fig-1: **Vinculin and other protein interactions**

In recent years there have been a great interest about Vinculin and its importance for cancer. It was observed that loss of Vinculin causes development of cancer [3-9]. Results of studies showed that Vinculin is a biomarker for a lot of type of cancer and it should be monitored in all section of cancer development.

Today some analytical method is used for protein detection like Elisa and Western blot [10]. Also

new technology proteomics studies have been used to observe proteome structure of cells. Among these methods LC/MS based proteomics has a special interest. Because LC/MS based methods give high sensitivity, accuracy and offer an opportunity to study with low amount of sample [11]. The other methods are more time consuming and less effective.

Nano LC is the main tool because of high sensitivity but system has some drawbacks like high backpressure that affects reproducibility and robustness [12]. UPLC is very good alternative for proteomics studies. UPLC/MS are so reproducible and robust system [13].

In this study we used UPLC/Q-TOF system to analyze caco-2 cell line proteome. One of the main goals was to identify vinculin. We made three replicate and we identified 5 unique peptides that belonged Vinculin. Also we used label free quantification method and calculated intensity of Vinculin.

MATERIALS AND METHODS

Cell Culture

Caco-2 colon cancer cells were first grown in 75 cm2 flasks in a MEM-Eagle medium containing 20% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acid, 3% sodium bicarbonate, 1% Glutamine, 1% penicillin-streptomycin. This process was continued until the number of cells sufficient for proteomic analysis.

After cell culture studies, Caco-2 colon cancer cell lines were washed in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 120 mM NaCl, pH: 7.4), washing with DPBS (138 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 7.4) dissolved and homogenized. The homogenate was centrifuged (14,000 g × 14 min at 4 ° C) and the precipitate (nuclear fraction and mitochondrial fraction) formed after centrifugation was discarded. The remaining solution was centrifuged again (100,000 g × 1 hour, 4 ° C) in an ultracentrifuge.

Sample Preparation UPLC/Q-TOF Analysis of Proteins

We used methanol, chloroform and water mixture for protein extraction from cell. This mixture gave us an opportunity to separate polar metabolites, lipids and proteins. Proteins were separated from this mixture and left at room temperature for 15 minutes to completely remove the methanol and chloroform from proteins.

The protein pellet was dissolved in 100 mM ammonium bicarbonate (20% methanol (v / v)) solution. In the dissolution process, the samples were left in the sonicator for about 30 minutes. Subsequently, 200 µM DTT was added reduce the proteins. Incubation with DTT continued for 15 min at 56 ° C. For alkylation of proteins, 100 mM iodoacetamide was added. In the dark, samples were incubated for 30 min at room temperature. After these treatments, the trypsin enzyme was added at a ratio of 1:50 (w/w), thereby cleaving the proteins. During this process the temperature was set at 37 ° C. The duration of incubation was 18 hours. After 18 hours, the resulting peptide mixture was left in a vacuum centrifuge to allow the ammonium bicarbonate to be evaporated. After vacuum centrifugation, peptide mixture was dissolved in (v/v) acetonitrile containing 0.1% formic acid. As a result of the treatment, the solution was obtained so that the protein concentration was 4 mg / mL protein concentration. The peptide mixtures thus obtained were prepared for UPLC/MS analysis.

UPLC Analysis

Peptides were separated with their polarity properties by using C18 column (zorbax C18 column 150x2.1mm, 1.8um, 300A). We used Agilent HPLC-1290 system. Acetonitrile and water, which contain 0,1 % (v/v) formic acid as an ion pairing agent was used as mobile phase. 150 minutes was used to separate peptides (Table 1).

Table-1: **Gradient for peptide separation**

Time (Min.)	Mobile phase B (%)	Mobile phase A (%)
0	3	97
130	45	55
135	85	15
136	85	15
138	3	97
150	3	97

Mass Spectroscopy Analysis

After separation peptides were ionized positively in ESI source. We study with positive mode and we adjust capillary voltage 4500 V to observe good spray. Also we selected temperature 350 ^{o}C. Ionized peptides were analyzed with Q-TOF/MS system. We used auto MS/MS mode above 1500 Count. We selected 45 V as collision energy.

Maxquant Analysis

For protein identification we used Maxquant bioinformatics tool. We tried to match recorded peptide MS/MS data with in silico MS/MS data to identify

amino acid sequences. We selected 20 ppm mass tolerance for matching process.

As a constant modification, carboamidomethylation on cysteine and oxidation on methionine as alternating modification and acetylation on -N units of proteins were chosen. During the identification of peptides, the smallest possible peptide length is thought to be 6, and the longest peptide length is thought to stem from 40 amino acids. In addition, the system is programmed to eliminate the heavier peptides from the 4600 Daltons. In the qualitative analysis of peptides, the FDR value was selected as 0.01. The FDR value for proteins was set at 0.01. Scores of 10 or more were accepted for modified peptides. It is desirable for a protein to have a score of at least 10 to identify proteins. For protein identification Human Uniprot database was used. For protein identification at least 2 unique peptides belong to identified protein was to observe at least two injection.

RESULTS

We separated peptides with UPLC system by using C18 column. Peptides wereionized in ESI source and then analyzed with Q-TOF-MS system. Total ion chromatography of peptide was showed in Figure 2.

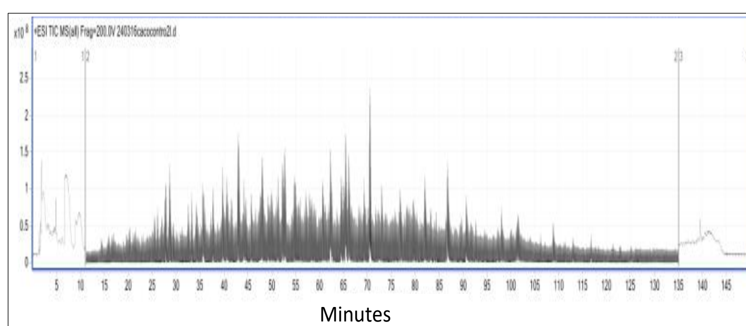

Fig-2: Total ion chromatography of caco-2 cell line proteome

The recorded MS/MS data was evaluated with Maxquant. hundreds of proteins were identified by matching uniprot Human database system. Among these proteins Vinculin protein was also identified. 5 unique peptides that belonged to Vinculin were identified. Identification of peptides was made by matching experimental MS/MS data with database. In Fgure3-7 It was showed that experimental and in silico MS/MS data of peptides. For peptide identification amino acid matching was the first stage. 20 ppm mass tolerance was used for amino acid sequence identification. In Figure 3-7 matched amino acid sequences was showed in MS/MS spectrum. Amino acid sequences was showed in red and blue line that was the C and N terminus of peptides. Identified peptides covered %9.6 of Vinculin. We made three replicate (three injection) for proteomics research. In every injection we observed 5 peptides.

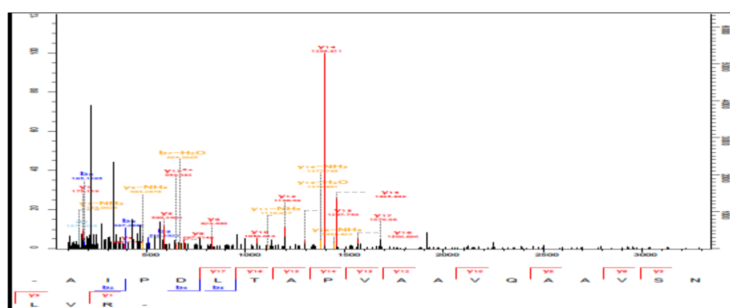

Fig-3: **MS/MS data of AIPDLTAPVAAVQAAVSNLVR and identified amino acid sequences**

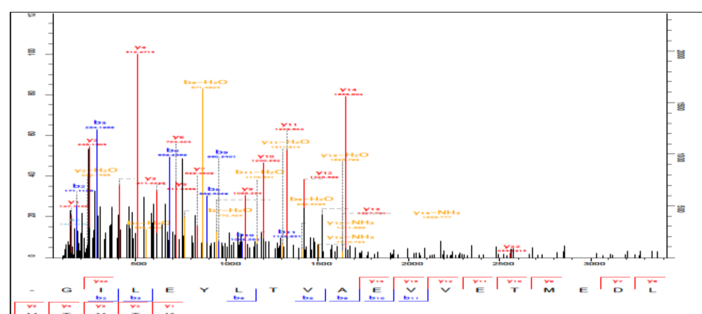

Fig-4: **MS/MS data of GILEYLTVAEVVETMEDLVTYTK and identified amino acid sequences**

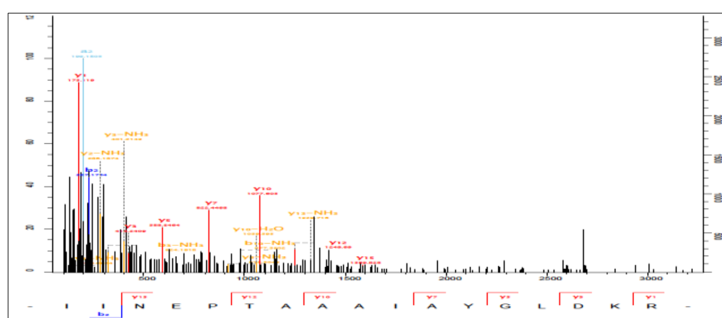

Fig-5: **MS/MS data of IINEPTAAAIAYGLDKR and identified amino acid sequences**

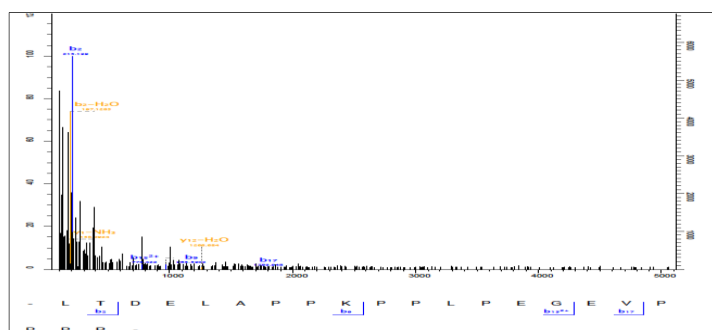

Fig-6: **MS/MS data of LTDELAPPKPPLPEGEVPPPR and identified amino acid sequences**

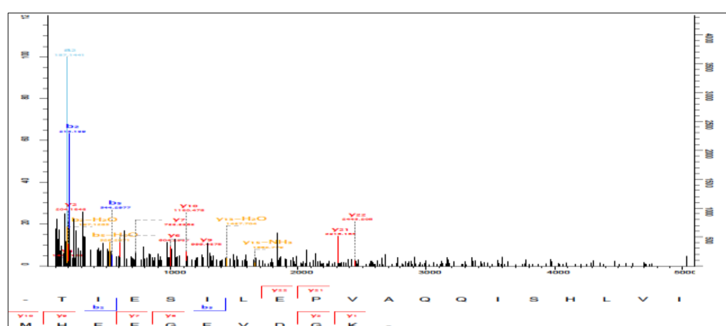

Fig-7: **MS/MS data of TTIESILEPVAQQISHLVIMHEEGEVDGK and identified amino acid sequences**

We used label free quantification method to compare Vinculin protein intensities between three different injection and to see reprocubility of UPLC/MS method. Protein intensity was calculated 438240 for first injection, 434740 for second injection and 433150 for last injection. Relative standart deviation was calculated as a % 0.5

DISCUSSIONS

Main goal of this study was to analyze caco-2 colon cancer cell line proteome. We tried to identify maxumum number of proteins but especially cancer markers like Vinculin. We evaluated MS/MS data and tried to identify Vinculin and We identified 5 unique peptide that belonged to Vinculin. This is acceptable for protein identification. All amino acid sequences we used to peptide identification were identified within 20 ppm mass tolerance Also we made three injection and we identified same 5 peptides in each injection. This results showed us our method is so reproducible for Vinculin identification. Also We calculated protein intensity with label free quantification method and our results were very satisfactorily good. Relative standart deviation of protein intensity was calculated a % 0.5. This is especially important for clinical studies to monitor Vinculin level in body fluids of cancer patients.

CONCLUSION

Vinculin is a protein marker and there is common interest about this protein. Some analytical system has been used to identify and quantify proteins in cell,tissues and biological fluids. Fort his purpose LC/MS based proteomics is the main tool today because system gives opportunity to analyze hundreds of proteins with high sensivity and accuracy in short time.

In this study we used UPLC/MS method for proteomics. We identify 5 unique peptide that belonged to Vinculin in each injection. Also We compare Vinculin intensity between injections and we observed that our method is so reproducible. Our method suggested that we can use UPLC/MS method for Vinculin protein identification and quantification *in vitro* and maybe *in vivo* studies.

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