

RESEARCH ARTICLE

Differential Protein Expression Profile Between CD20 Positive and Negative Cells of the NCI-H929 Cell Line

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Abstract

At present, multiple myeloma (MM) remains an incurable disease and clonogenic cells may be responsible for disease relapse. It has been proposed that CD20+/CD138- NCI-H929 cells could be hallmarks of MM clonogenic cells. Here, the immunology phenotype of NCI-H929 cells is described. Only a small population of CD20+/CD138- cells (<1%) was found in the NCI-H929 cell line, but CD20+/CD138- cells were not detected. We found that CD20+/CD138+ cells were able to exhibit clonogenic capacity by colony formation assay and continuous passage culture. Proteins were analyzed by 1D-SDS-PAGE and TMT based quantitative differential liquid chromatography tandem mass spectrometry (LC-MS/MS). 1,082 non-redundant proteins were identified, 658 of which were differentially expressed with at least a 1.5-fold difference. 205 proteins in CD20+ cells were expressed at higher levels and 453 proteins were at lower levels compared with CD20- cells. Most proteins had catalytic and binding activity and mainly participated in metabolic processes, cell communication and molecular transport. These results proved that there are different biological features and protein expression profile between CD20+ and CD20- cells in the NCI-H929 cell line.

Keywords: Multiple myeloma - NCI-H929 cells - proteomics - mass spectrometry

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Introduction

The survival of multiple myeloma (MM) patients has been improved by the usage of high-dose therapy and the novel agents, but this disease is still incurable (Khan et al., 2006; Kyle et al., 2008; Raab et al., 2009; Rajkumar et al., 2011). Increasing data suggest that the relapse of MM is driven by clonogenic cells which have tumorigenic and self renewal properties (Brennan et al., 2009). It has been found that CD20+/CD138- may be the hallmark of clonogenic cells in NCI-H929 cells (Matsui et al., 2004; Huff et al., 2008; Matsui et al., 2008). So, it is hypothesized that MM clonogenic cells origins from CD138- B cells. However, the phenotype of clonogenic myeloma cells remains a matter for debate. It was showed that CD20+ and CD138- cells were not the mark of clonogenic cells (Jakubikova et al., 2011; Chiron et al., 2012; Hosen et al., 2012). Moreover, some study found that NCI-H929 cells did not contain CD20 positive cells and suggested that CD20 positive cells were not associated with a cancer stem cell phenotype (Paño et al., 2012).

In this study, we found the presence of CD20+ cells in NCI-H929 cells and the difference of biological features and protein expression profile between CD20+ and CD20- cells. It indicated that CD20+ cells were able to be found

in NCI-H929 cells and had differential protein expression profile from CD20- cells.

Materials and Methods

Cell line and cell culture

NCI-H929 cell line (Beijing Sunbio Biotech) was incubated in RPMI 1640 media consisting of 2 mM L-glutamine and 10% fetal bovine serum at 37 °C and 5% CO₂. MACS cell separation system (Miltenyi Biotec) was used to separated cells. CD20+ or CD20- subsets were isolated from NCI-H929 cells using mouse antihuman CD20 antibodies coupled to magnetic microbeads (Miltenyi Biotec) followed by magnetic column selection (Miltenyi Biotec).

Colony formation assay

Logarithmically growing cells were suspended into single cells and were plated in 1 mL RPMI 1640 media consisting of 0.9% methylcellulose, 30% fetal bovine serum and 2 mM L-glutamine with the concentration of 1×10^3 cells/mL at 37°C and 5% CO₂. After 14 days of growth, colonies consisting of more than 40 cells were scored. Then, clonogenic cells were washed using RPMI 1640 media and plated in RPMI 1640 media consisting

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of 0.9% methylcellulose as described.

Flow cytometry assay

Cells were suspended in phosphate-buffered saline (PBS) and stained with CD20 (PE conjugated mouse antihuman) and CD138 (APC conjugated mouse antihuman) antibodies (BD Biosciences) for 30 minutes at 4°C. Cells were washed then resuspended in PBS and analyzed on a FACS Vantage SE flow cytometer (BD Biosciences). Cells were analyzed by gating on CD20 or CD138 populations and subsequently evaluating immunological phenotype of cells.

1D-SDS-PAGE and mass spectrometry

Total proteins of CD20+ and CD20- cells were extracted from cells using Total Protein Extraction Kit (Beijing Biosynthesis Biotechnology). Protein samples were separated by 12% one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE) and stained with Coomassie Brilliant Blue R250 solution (Beijing Biosynthesis Biotechnology). Protein zones were manually excised from the gels. Then, gel pieces were destained and dehydrated with acetonitrile. Subsequently, the proteins were reduced with DTT, alkylated with iodoacetamide, and incubated with 12.5 µg/µl sequencing grade trypsin (Promega, Madison, WI) at 37°C for 12h. For protein quantization, peptides of CD20+ and CD20- cells were respectively labeled with TMT 126 and 127 (Thermo, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instruction. Briefly, the TMT reagents were dissolved by anhydrous acetonitrile. The labeling reaction was carried out by incubation of tryptic peptides with the TMT reagents for 1 hour at room temperature, and the reaction was quenched using hydroxylamine. The TMT-labeled peptides were desalted using the stage tips. After the labeling, the peptides were extracted twice with 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/50% acetonitrile, respectively, and dried in a vacuum centrifuge. The volumes of the extraction were adjusted to 15 µl with 0.1% trifluoroacetic acid, of which 5 µl was analyzed by LC-MS/MS.

For LC-MS/MS analysis, each digestion product was separated by a 65-min gradient elution at a flow rate 0.25 µl/min with the EASY-nLCII™ integrated nano-HPLC system (Proxeon, Denmark) which is directly interfaced with the Thermo LTQ-Orbitrap mass spectrometer. The LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur software. The experiment consisted of a single full-scan mass spectrum in the Orbitrap (400–1,800 m/z, 30,000 resolutions) followed by 20 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy (CID). The MS/MS spectra from each LC-MS/MS run were searched against the selected database using an in-house Proteome Discovery searching algorithm (Hong et al., 2011).

Bioinformatics analysis of proteins

The MS/MS peak lists were searched against the IPI human database using SEQUEST software. The search criteria were as follows: full tryptic specificity

was required; one missed cleavages were allowed; carbamidomethylation was set as fixed modification; the oxidation (M) was set as variable modification; precursor ion mass tolerances were set at 10 ppm for all MS acquired in the Orbitrap mass analyzer; and the fragment ion mass tolerance was set at 0.8 Da for all MS2 spectra acquired in the linear ion trap (Hong et al., 2011). The proteins data were also globally analyzed using an online analysis tool, PANTHER (<http://www.pantherdb.org>), by submitting IPI access number of all identified proteins. A protein-protein interaction network was done by STRING software through inputting IPI number (<http://string.embl.de>) (Zhang et al., 2011).

Statistical analysis

Statistical analysis was carried out using the software package SPSS version 17.0. Data are expressed as mean ± SEM. Differences were assessed using one-way ANOVA and a significance level of P<0.05 was required.

Results

Expression of CD20 and CD138 in NCI-H929 cells

All of the NCI-H929 cells expressed high levels of CD138 antigens. CD138- cells were not detected by flow cytometric analysis and can not be isolated from NCI-H929 cells by MACS cell separation system. NCI-H929 cells contained a distinct population of CD20+ cells that represented less than 1% of total cells (Figure 1A,

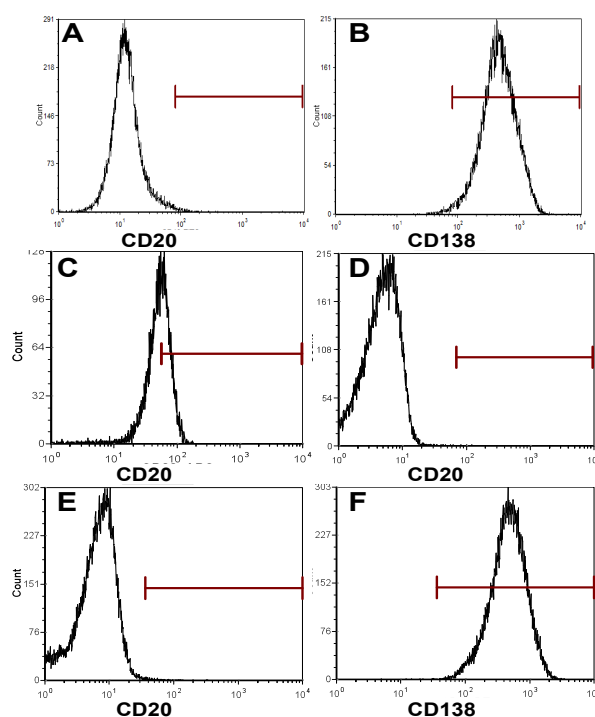


Figure 1. Flow Cytometric Evaluation Antigen Expression of CD20 and CD138 in NCI-H929 Cells. (A) Expression of CD20 in NCI-H929 cells. (B) Expression of CD138 in NCI-H929 cells. (C) Expression of CD20 in CD20+NCI-H929 cells isolated by MACS cell separation system. (D) Expression of CD20 in CD20-NCI-H929 cells isolated by MACS cell separation system. (E) Expression of CD20 in CD20+NCI-H929 cells 14 days after sorting. (F) Expression of CD138 in CD20+NCI-H929 cells 14 days after sorting

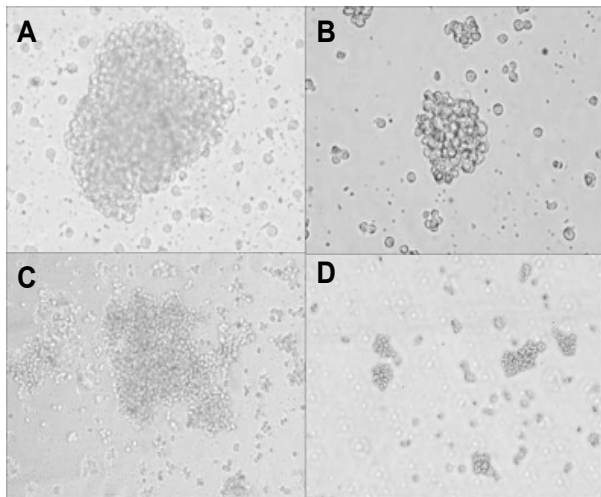


Figure 2. Clonogenicity of NCI-H929 Cells by CD20 Expression. (A) Clonogenic expansion of CD20+ cells. (B) Clonogenic expansion of CD20- cells. (C) Clonogenic expansion of CD20+ cells after continuous passage culture. (D) Clonogenic expansion of CD20- cells after continuous passage culture

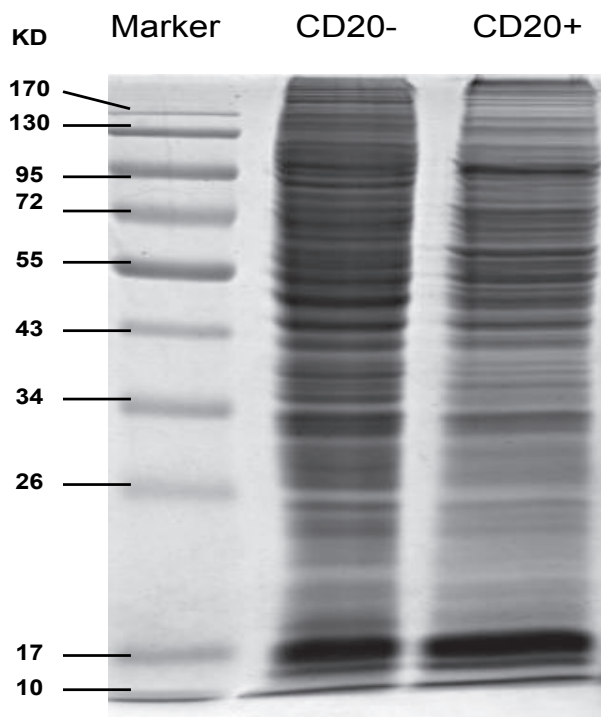


Figure 3. SDS-PAGE (12%) of the Protein from CD20+ and CD20- Cells. The gel was stained with Coomassie Brilliant Blue R250

B). CD20+ and CD20- subsets were isolated by MACS cell separation system and subsequent flow cytometric analysis demonstrated that CD20+ cells were able to be completely separated from NCI-H929 cells (Figure 1C, D).

Biological features of CD20+ cells

To examine the clonogenic capacity of CD20+ cells, separated cells were plated into RPMI 1640 media consisting of 0.9% methylcellulose. CD20+ cells were able to form more colonies which contained more cells compared with CD20- cells (Figure 2A, B). After continuous passage culture for 5 times, there were still few CD20+ cells remaining the ability to forming colonies.

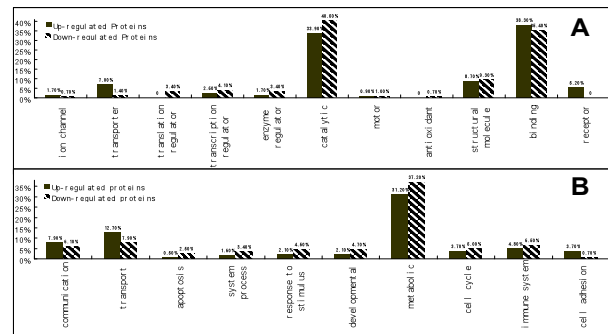


Figure 4. Bioinformatic Analysis of Differentially Expressed Proteins. A. Function distribution graph of identified differential proteins. B. Biological processes participated by identified differential proteins. PANTHER database were used for the data analysis

However, CD20- cells lost colony forming ability after continuous passage culture (Figure 2C, D). To examine differentiation potential, CD20+ cells were analyzed the expression of CD20 antigen 14 days after sorting. The majority of CD20+ lost the expression of CD20 antigen and highly expressed CD138 antigen (Figure 1E, F).

Identification of differentially expressed proteins

Total proteins were isolated from CD20+ and CD20- cells and separated in SDS-PAGE gels which subsequently were stained with Coomassie Brilliant Blue R250. Each of the two stained polyacrylamide gel were divide into ten pieces (Figure 3). LC-MS/MS identification revealed that 1082 proteins were identified, which had a p-values > 95% confidence level (ProtScore > 1.8) and at least more than one peptide above the 95% confidence level. 48% (519 of 1082) proteins were identified by more than 5 peptides, 18% (200 out of 1082) by 4 peptides and 34% (363 out of 1082) by 2 peptides. Cutoff TMT ratios of fold-change for protein expression were >1.5 for up-regulation and <0.67 for down-regulation. 658 differentially expressed proteins were screened including 205 proteins up-regulated in CD20+ cells and 453 down-regulated in CD20+ cells.

Bioinformatic analysis of differentially expressed proteins

Among 205 proteins up-regulated and 453 proteins down-regulated, 132 and 231 proteins were respectively recognized and analyzed by PANTHER Protein Classification System. According to the annotations from PANTHER database, differential proteins were involved in catalytic activity, binding activity, structural molecule activity, ion channel activity, transporter activity, translation regulator activity, transcription regulator activity, enzyme regulator activity, motor activity, antioxidant activity and receptor activity. Most proteins had catalytic activity and binding activity. The ratio of proteins was high which had the activity of ion channel, transporter, binding and receptor among differential proteins up-regulated compared with down-regulated proteins. However, the ratio of proteins was low which had the activity of translation regulator, transcription regulator, enzyme regulator, catalysis and antioxidation (Figure 4A). Roles of differential proteins in biological processes mainly included cell communication, transport, apoptosis, system process, response to stimulus, developmental

process, metabolic process, cell cycle, immune system process and cell adhesion. Most proteins participated in metabolic process, cell communication and transport. The ratio of proteins was high which participated in processes of cell communication, transport and cell adhesion among differential proteins up-regulated compared with down-regulated proteins. However, the ratio of proteins was low which participated in other processes (Figure 4B).

Discussion

MM is characterized by the clonal proliferation of malignant plasma cells. It was proved that malignant plasma cells were not the root of this disease and the clonogenic ability of memory B cells induced the relapse of MM (Pilarski et al., 2000; Pilarski et al., 2002). CD20+/CD138- may be the hallmark of CSC in NCI-H929 cells (Matsui et al., 2004; Matsui et al., 2008). However, other cells also had tumorigenic and self renewal properties (Yaccoby, 1998; Yaccoby et al., 1999). Moreover, CD138- cells were only found in few human myeloma cell lines and were not relevant to the clonogenicity (Jakubikova et al., 2011). It was found that CD138- clonogenic cells are plasma cells rather than B cells, and that MM plasma cells including CD138(-) and CD138(+) cells have the potential to propagate MM clones in vivo in the absence of CD19(+) B cells (Hosen et al., 2012). In patients, it was believed that CD20-CD138+ was the origin of clonogenic myeloma cells (Chiron et al., 2012). Moreover, some researchers could not detect CD20 positive cells in NCI-H929 cells and believed that CD20 positive cells were not associated with a cancer stem cell phenotype (Paño et al., 2012). At present, the nature of clonogenic myeloma cells remains unclear.

In this study, we showed that all of NCI-H929 cells expressed high levels of CD138 antigens. CD138- cells were not detected by flow cytometric analysis and were not able to be isolated from NCI-H929 cells by MACS cell separation system. While CD20+ cells were successfully separated from NCI-H929 cells. It suggested that CD20+ cells existed in NCI-H929 cells and its proportion was very low.

We also found that CD20+ cells were able to form more and larger colonies compared with CD20- cells by colony formation assay. After several generations, some of CD20+ cells were still able to keep clonogenic capacity, but CD20- cells lost colony forming ability. Moreover, CD20+ cells had differentiation potential which could generate CD20- cells by passage culture. The results suggested that biological features of CD20+ cells were different from CD20- cells.

In order to identify more membrane proteins, TMT isotope labeling, in conjunction with tandem MS was used. TMT is a high content proteomic technique for substrate degradomics, and has been widely used in proteomic research. In this study, 1082 proteins were identified and 658 differentially expressed proteins were screened including 205 up-regulated proteins and 453 down-regulated proteins in CD20+ cells. Expressed profile of CD20+ cells was different from CD20- cells. Differential proteins were mostly involved in catalytic

activity and binding activity according to the annotation from PANTHER database. Functions of up-regulated proteins mainly included ion channel, transporter, binding and receptor; however, functions of down-regulated proteins mainly included activity of translation regulator, transcription regulator, enzyme regulator, catalysis and antioxidation. It may be relevant to the biological features of CD20+ cells. For biological process, most differential proteins participated in metabolic process, cell communication and transport. Moreover, up-regulated proteins mainly participated in processes of cell communication, transport and cell adhesion; down-regulated proteins mainly participated in other processes including apoptosis. These changes may be the reason why CD20+ cells had different biological features from CD20- cells.

In conclusion, CD20+ cells existed in NCI-H929 cells and its biological features were different from CD20- cells. Moreover, expressed profile of CD20+ was different from CD20- cell. Different biological features of CD20+ cells may be induced by expression changes of proteins in cells.

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