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Original article

Expression and pathogenesis of VCAM-1 and VLA-4 cytokines in multiple myeloma

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ABSTRACT

Objective: The objective of this study is to investigate the expression of Vascular cell adhesion molecule-1 (VCAM-1) and very late appearing antigen-4 (VLA-4) cytokines in MM (multiple Myeloma).
Method: Forty patients with MM are selected as the experimental group and 30 healthy persons as the control group. Flow cytometry is used to detect the expression of VCAM-1 (CD106), VLA-4 (CD49d), CD38 and CD138 antigens in experimental group and control group. ELISA (Enzyme Linked Immunosorbent Assay) is used to detect the concentration of VCAM-1 in serum of experimental group and control group. RT-PCR is used to detect the expression of VCAM-1.
Results: The positive rate and antigen expression rate of VACM-1 antigen in the experimental group were significantly higher than those in the control group ($P < 0.05$). There were statistical differences of VLA-4 and VCAM-1 antigens between the initial diagnosis group and the relapse/refractory group, and between the relapse/refractory group and the platform stage group ($P < 0.05$). There were significant differences between VLA-4 antigen and VACM-1 antigen, phase I and phase II, and between phase I and phase III ($P < 0.05$). The concentration of VCAM-1 and the expression of VCAM-1 mRNA in the experimental group were significantly higher than ($P < 0.01$). In the different stages of ISS (International Staging System) and different disease groups in the experimental group, the concentration of VCAM-1 and the expression level of VCAM-1 mRNA are significantly different among the three groups of stage I, II and III ($P < 0.01$). There is a significant difference between the initial diagnosis group, the relapse/refractory group and the platform group ($P < 0.05$).
Conclusion: There are abnormal expressions of adhesion molecules VCAM-1 and VLA-4 in multiple myeloma patients, which are related to ISS staging.

0. Introduction

Multiple Myeloma (MM) is a malignant plasmacyte tumor (Belloni et al., 2018) characterized by malignant proliferation of abnormal plasmacytes in bone marrow (Xu et al., 2018), presence of monoclonal immunoglobulin in serum and urine, suppression of normal immunoglobulin and osteolytic lesions (Khalife et al., 2019). It has been proved that Bone marrow microenvironment plays an important role in its pathogenesis (Terpos et al., 2018; Kawano et al., 2015). Most scholars believe that the pathogenesis of MM may be closely related to the activation of some tumor genes, the abnormal increase of some cytokines, or the abnormal expression of cell adhesion molecules (Anreddy and Hazlehurst, 2017). VCAM-1, or CD106, is a member of the immunoglobulin super family and is produced by the expression of vascular endothelial cells (Terpos et al., 2019). In MM, adhesion molecules are not only closely related to its pathogenesis, but also an important factor in the increase of thrombosis in patients with myeloma (Ghobadi et al., 2018). The receptor for VCAM-1 is VLA-4 (Brunetti et al., 2017), or CD49d. VCAM-1/VLA-4 can mediate the involvement of leukocytes in the process of histopathological changes and chronic inflammation (Tenreiro et al., 2017). With

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the increasing expression of VCAM-1, neovascularization areas can appear in the lesions. Clinically, CD38/CD138 gate-setting strategy is often used to screen multiple myeloma cells, in order to achieve the purpose of screening and purifying myeloma cells. It is widely used in clinical diagnosis and research (Sahin and Demirer, 2018; Kün-Darbois et al., 2017).

To further study the pathogenesis of multiple myeloma to guide clinical diagnosis and treatment, in this study, the expression levels of VCAM-1 (CD106) and VLA-4 (CD49d) in experimental group and control group are detected by using CD38/CD138 gate-setting strategy, and their differences in different stages of ISS in MM patients are explored. This study is of great theoretical significance to elucidate the pathogenesis of multiple myeloma, and provide powerful experimental data to support the diagnosis and treatment.

1. Research methods

1.1. Research object and grouping

The study subjects of flow cytometry mainly come from 40 patients with MM from October 2017 to February 2019. They are taken as the experimental group. They are divided into 20 patients who are initially diagnosed, 9 patients who are reexamined or refractory, and 11 patients who reach plateau stage after chemotherapy. According to ISS staging criteria, there are 11 cases of stage I, 13 cases of stage II and 16 cases of stage III. 30 healthy people in the Hospital are selected as the control group. According to the stage standard of MM efficacy response evaluation, the patients in the experimental group were divided into three groups: the first diagnosis group, the relapse/refractory group, and the platform stage group. The relapse/refractory group included the patients with multiple myeloma with the disease progression (PD), the stable disease (SD), and the minimal remission (MR) of relapsed refractory myeloma. The platform stage group was multiple myeloma patients with partial release (PR), very good partial remission (VGPR), strict complete remission (SCR), and complete remission (CR). The study was reviewed and approved by the hospital ethics committee, and all subjects signed the informed consent.

Inclusion criteria: all patients met the diagnostic criteria of multiple myeloma; blood samples were provided and cytokines were tested.

Exclusion criteria: hemodialysis patients; patients with end-stage disease, estimated survival time within 3 months; patients with other serious diseases such as malignant tumor; pregnant patients.

1.2. ISS standard and MM response evaluation staging standard

The criteria of ISS stage are as follows: stage I: serum β2-macroglobulin <3.5 mg/L; albumin ≥35 g/L; stage II: not meeting stage I and III; stage III: serum β2-microglobulin ≥5.5 mg/L.

The evaluation criteria of MM efficacy response were as follows: (1) PD: hypercalcemia associated with abnormal proliferation of plasma cells; the absolute value of plasma cells was higher than 10%; the increase of "M protein" was at least 25%; if "M protein" was not detected, the absolute value of free immunoglobulin light chain (FLC) was ≥100 mg/L; soft tissue plasmacytoma increased by >25%, or new osteolytic changes appeared. The diagnosis was consistent with at least one of the above items. (2) SD: there was no new or original osteopathy, and it did not meet the standards of PR, PD, CR and VGPR. (3) MR: 25%≤M protein in serum <49%, M protein in urine decreased to 50–89%/24 h. (4) PR: the serum "M protein" decreased to no <50%, the urine "M protein" decreased to no <90%, and the urine "M protein" content was <200 mg/24 h. (5) VGPR: there was no "M protein", but immunofixation electrophoresis in blood and urine could be positive. Or the "M protein" in serum decreased by 90%, and the "M protein" in urine was <100 mg. (6) SCR: the "M protein" detected in blood or urine was negative; FLC ratio was within the normal range, and it was proved that there was no monoclonal plasma cell by two methods simultaneously. (7) CR: the "M protein" detected in blood or urine was negative. If "M protein" was not detected, the ratio of FLC should be between 0.26 and 1.65.

1.3. Flow cytometry

The bone marrow of the experimental group and the control group are collected aseptically and injected rapidly into the test tube containing EDTA (Dulbecco's Modified Eagle Medium) anticoagulant. After several times of slow inversion and homogenization, flow-cytometric fluorescent antibodies are added for detection. The steps are as follows:

The flow tube is prepared and numbered, and antibodies are added. After adding EDTA anticoagulant bone marrow samples, it is necessary to shake and mix well and avoid light for 20 min at room temperature. The erythrocyte lysate Opti Lyse C is added to destroy the erythrocyte membrane and shake well. Incubation is conducted at room temperature for 10 min. PBS buffer 2 mL is added and mixed well. After centrifugation for 5 min at 1500 rpm, the supernatant is discarded and mixed to vibrate. After adding polyformaldehyde solution to fix it, it needs to be tested on the computer immediately.

No <60,000 cells are obtained from each sample and analyzed by FCSExpress software. Through the gate strategy of CD45/SSC combined with CD38/CD138, the cell population to be detected is set up, the antigen expression of the cell population is analyzed, and the monoclonal antibodies including CD138, CD38, CD106 and CD49d are detected.

1.4. Enzyme-linked immunosorbent assay

Fasting venous blood 5 mL is drawn from patients in the study group. After blood coagulation, 3000 rpm is centrifuged for 5–10 min. The upper serum is absorbed into EP tube and stored at −70 °C. The steps are as follows:

Serum specimens are taken out. According to the instructions of the VCAM-1 kit, preparations are made before the experiment. The standard is diluted. The blank hole and the sample hole to be measured are set. In the pore of the sample to be tested, 40 μL diluent of the sample and 10 μL of the serum sample (diluted 5 times) is added. No sample is added to the blank pore. The coated plate is sealed and incubated at 37 °C for 30 min. The sealing film is removed and the liquid is discarded. Fully automatic washing machine is used to wash five times, and finally to dry. Each pore is added with enzyme-labeled reagent solution of 50 μL. The coated plate is sealed and incubated at 37 °C for 30 min. The sealing film is removed and the liquid is discarded. Fully automatic washing machine is used to wash five times, and finally to dry. Each hole is added with the chromogenic reagents A and B in turn to shake even. After colouring at 37 °C for 15 min, 50 μL termination solution is added to each pore to terminate the reaction. The color changes from blue to yellow. OD (optical density) values of each hole are measured at 450 nm.

1.5. Detection of VCAM-1 mRNA expression

Total RNA is extracted from cells by Trizol Company and OD value is measured to quantify RNA concentration. Olig (dT) 15 is used as a primer and RNA reverse transcription kit

(TOYOBO Company) is used to prepare the DNA. The DNA was used as a template. The total reaction system is 20 μL according to the operation of Realtime PCR Master Mix instructions. The reaction conditions are set as follows: after pre-denaturation for 30 s at 95 °C, denaturation for 5 s at 95 °C, annealing for 5 s at 56 °C, and elongation for 15 s at 72 °C. According to Livak and Schmittgen's comparison threshold method, the CT value is read. The expression of VCAM-1 is calculated by equation $2^{-\triangle\triangle Ct}$.

1.6. Statistical methods

SPSS 22.0 software is used to analyze the experimental data. The mean ± standard deviation is used for each group. The comparison of counting data is tested by χ^2. The difference between the two groups is analyzed by *t*-test of independent samples. The correlation between the two variables is analyzed by Pearson correlation analysis (linear correlation analysis). $P < 0.05$ shows significant difference with statistical significance.

2. Result

2.1. Test results of antigens in experimental group and control group

The results show that the positive rates of CD38, CD138 and CD49d in the experimental group are not significantly different from those in the control group ($P > 0.05$). However, the positive rate of CD106 antigen in the experimental group is 62.50% (25/40). Compared with the control group, whose positive rate is 90.00% (27/30), the difference is significant ($P < 0.05$). The expression levels of CD38 and CD49d antigens are strongly positive. There is no significant difference in antigen expression between the two groups ($P > 0.05$). There is no significant difference in the expression rate of CD138 antigen between the two groups ($P > 0.05$). The expression rate of CD106 antigen is (22.48 ± 14.36%), which is significantly different from that of the control group (64.33 ± 19.81%) ($P < 0.01$), as shown in table 1 and Fig. 1 specifically:

2.2. Detection results of antigens in different disease groups of MM

According to the state of disease at the time of enrollment, the experimental group is divided into three groups: initial diagnosis group, relapse/refractory group and platform group. The specific expression of antigen in each group is shown in Table 2 and Fig. 2. Compared with the initial diagnosis group, CD49d and CD106 in the relapse/refractory group were significantly lower ($P < 0.05$), while CD38 and CD138 were not significantly different ($P > 0.05$). Compared with the relapse/refractory group, CD49d and CD106 in the platform group were significantly higher ($P < 0.05$). There was no significant difference between the initial diagnosis group and the platform group ($P > 0.05$).

2.3. Detection results of MM antigens in different stages of ISS

According to the ISS stage of the subjects, the experimental group is divided into three groups: stage I, stage II and stage III. The expression rates of related antigens in each group are shown in Table 3 and Fig. 3. Compared with stage II, CD49d and CD106 in stage I decreased significantly ($P < 0.05$), while CD38 and CD138 had no significant difference ($P > 0.05$). Compared with stage III, CD49d and CD106 in stage I decreased significantly ($P < 0.05$), while CD38 and CD138 had no significant difference ($P > 0.05$). There was no significant difference between the antigens of stage II and stage III ($P > 0.05$).

2.4. Serum VCAM-1 concentration level

The serum VCAM-1 concentration and VCAM-1 mRNA level of MM patients in the experimental group are significantly higher than those in the control group ($P < 0.05$), as shown in table 4 for VCAM-1 concentration and VCAM-1 mRNA level in stage I, stage II, stage III, initial diagnosis group, relapse/refractory group and platform group.

3. Discussion

Some studies have found that (Pietronigro et al., 2019) VCAM-1 can promote angiogenesis. In human atherosclerotic plaques, the expression of VCAM-1 is mostly in the neovascularized area (De Waal et al., 2017). VCAM-1 has chemotaxis to vascular endothelial cells and angiogenesis in cornea of mice. Some studies suggest that (Natoni et al., 2020) VCAM-1 can be used as a physiological indicator of neovascularization. It is also found that the concentration of serum VCAM-1 in breast cancer patients is significantly correlated with microvessel density (Choi et al., 2018; Tochigi et al., 2017), suggesting that the determination of serum VCAM-1 can be used as an alternative indicator of vascular proliferation in breast cancer. VCAM-1 can not only mediate inflammatory effects, but also promote angiogenesis, which also includes promoting angiogenesis of tumors, and providing abundant and sufficient blood for tumors, leading to their growth and metastasis (Al-Dhabi et al., 2020; Bergqvist et al., 2019; Lin et al., 2019).

As a ligand of VCAM-1, VLA-4 belongs to the integrin family of adhesion molecules. It mediates the adhesion between hematopoietic stem cells and bone marrow, and also participates in the redistribution and homing of bone marrow hematopoietic stem cells after transplantation (Qian et al., 2007; Minguell et al., 2000).

The results of this study show that VLA-4 is strongly expressed in MM patients. The positive rate of CD49d in the experimental group is 94.59%, and the expression rate of CD49d antigen is higher than that in the control group. It indicates that CD49d is involved in the process of adhesion between bone marrow cells and cell matrix in MM. The occurrence of MM is correlated with abnormal CD49d (Hatano et al., 2009). Relevant reports showed that the expression level of CD49d on the tumor cell surface of MM patients increased (Bingham et al., 2017), which is consistent with the results of this study. The serum concentration of VCAM-1 and the expression of VCAM-1 mRNA in MM patients are significantly higher than those in control group ($P < 0.05$). The concentration of VCAM-1 and the expression level of VCAM-1 mRNA in ISS staging groups increases from stage I to stage III. There are significant

Table 1
Positive rate and expression rate of each antigen in experimental group and control group.

Index		CD38	CD138	CD49d	CD106
Positive rate (n, %)	experimental group	40(100.00%)	37(92.50%)	38(95.00%)	25(62.50%)*
	control group	27(90.00%)	29(96.67%)	26(86.67%)	27(90.00%)
Expression rate (%)	experimental group	96.62 ± 7.15	86.56 ± 16.84	70.11 ± 19.65	22.48 ± 14.36**
	control group	97.43 ± 9.54	88.63 ± 17.16	67.26 ± 18.28	64.33 ± 19.81

Note: The experimental group is compared with the control group, *P < 0.05, **P < 0.01.

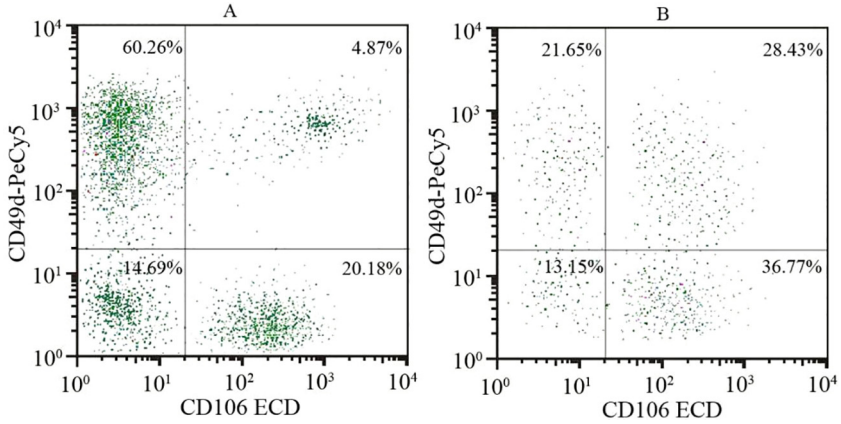

Fig. 1. Flow pattern of antigen expression in MM patients and controls (A: experimental group; B: control group).

Table 2
Comparison of antigen expression rates among different disease groups of MM.

Index (%)	CD38	CD138	CD49d	CD106
Initial diagnosis group	97.15 ± 3.65	92.65 ± 10.33	77.25 ± 16.26	25.41 ± 18.06
Relapse/refractory group	96.57 ± 10.34	85.14 ± 12.15	57.36 ± 15.65#	43.74 ± 19.37#
Platform group	94.07 ± 15.23	90.33 ± 13.17	74.02 ± 15.38&	24.08 ± 11.87&

Note: #, compared with the initial group, P < 0.05; &, compared with the relapse/refractory group, P < 0.05.

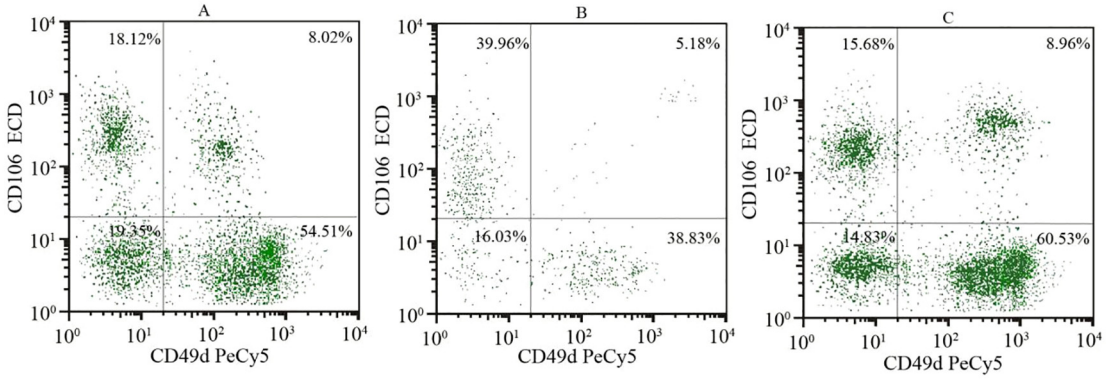

Fig. 2. Flow pattern of antigen expression in different disease stages of MM patients (A: initial diagnosis group; B: relapse/refractory group; C: platform group).

Table 3
Comparison of the expression rates of MM antigens in different stages of ISS.

Index (%)	CD38	CD138	CD49d	CD106
Stage I	94.28 ± 12.64	93.56 ± 9.86	68.73 ± 16.75#&	51.20 ± 16.17#&
Stage II	97.58 ± 16.91	86.41 ± 13.35	85.86 ± 19.06	33.37 ± 15.75
Stage III	91.24 ± 20.65	92.12 ± 15.16	87.28 ± 20.76	30.85 ± 13.54

Note: #, compared with stage II, P < 0.05; &, compared with stage III, P < 0.05.

differences among the three groups (P < 0.05). The concentration of VCAM-1 and the expression of VCAM-1 mRNA are the highest in the relapse/refractory group.

In conclusion, there are abnormal expressions of adhesion molecules VCAM-1 and VLA-4 in MM patients, which are related to ISS staging. Studying the expression of these adhesion molecules in patients with MM is of great significance for elucidating the pathogenesis of MM and guiding clinical diagnosis and treatment. However, there are also some shortcomings in the research process, such as the small amount of data collected from the samples, leading to a certain deviation of the results. Hence, in the later research process, the data capacity will be further increased, so that the results obtained are more valuable for reference.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

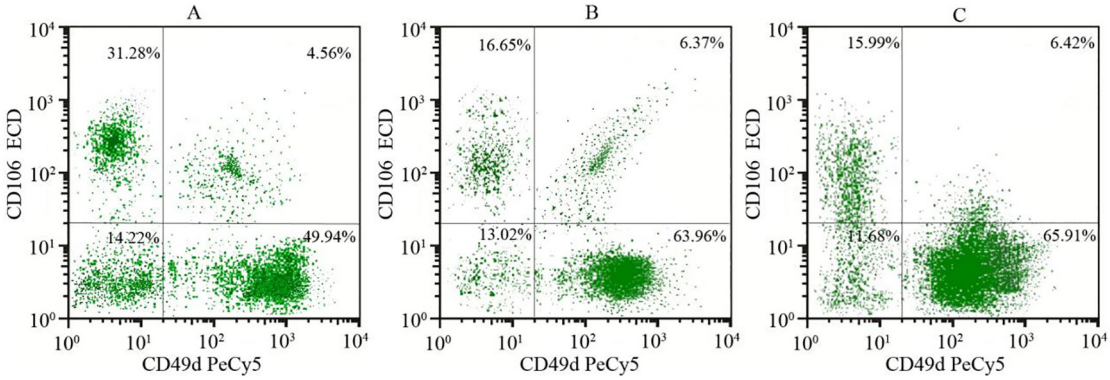

Fig. 3. Flow pattern of antigen expression in different stages of ISS in MM patients (A: ISS stage I group; B: ISS stage II group; C: ISS stage III group).

Table 4
Serum VCAM-1 concentration and mRNA level.

Grouping		Cases	VCAM-1 concentration	VCAM-1 mRNA level	P value
MM experimental group		40	911.84 ± 46.35	7.8 ± 0.66	P < 0.05
Control group		30	422.29 ± 31.85	1.00 ± 0.00	
ISS staging	Stage I	11	642.17 ± 37.74#	6.8 ± 0.51#	P < 0.05
	Stage II	13	809.56 ± 48.17*	7.2 ± 0.58*	
	Stage III	16	1038.48 ± 39.83&	8.8 ± 0.63&	
Disease grouping	Initial diagnosis group	20	908 ± 39.18a	7.9 ± 0.26a	P < 0.05
	Relapse/refractory group	9	1017 ± 94.83b	8.6 ± 0.38b	
	Platform stage group	11	712.97 ± 31.26c	5.9 ± 0.17c	

Note: Compared with stage I, &P < 0.01. Compared with stage II, #P < 0.01. Compared with stage III, *P < 0.01. Compared with relapse/refractory group, aP < 0.05. Compared with platform group, bP < 0.05. Compared with the initial group, cP < 0.05.

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