

Establishment of an Anti-HIV/AIDS Agents Screening Technique Based on Human CXCR4 Promoter

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Abstract — Objective: To construct an anti-HIV/AIDS agents screening system by handling human CXCR4 promoter with medicated serum. Methods: Human CXCR4 (CXC Chemokine Receptor 4) promoter gene was inserted into the reporter vector pGL4. Recombinant plasmid pGL4-CXCR4 was transfected into Jurkat cells (the cell line of acute T lymphocyte leukemia). The stable transfected cell were screened by G418. Thirteen kinds of traditional Chinese medicine were given to rats intragastrically and the medicated serum were collected. After the stable transfected cells were handling separately by medicated serum, CXCR4 promoter expression in the cells was detected. CXCR4 protein expression in change groups cells were tested by Western blotting analysis. Result: Cortex phellodendri chinensis and Herba houttuyniae can depress the activity of the transfected CXCR4 promoter in Jurkat cell, the luciferase activity of which is lower remarkably than of control group ($p < 0.05$). Herba lobeliae chinensis can enhance the activity of the transfected CXCR4 promoter in Jurkat cells, the luciferase activity of which is higher remarkably than that of the control group ($p < 0.05$). Conclusion: An anti-HIV/AIDS agents screening system based on Human CXCR4 promoter was constructed. Cortex phellodendri chinensis and Herba houttuynia were presumed to have the potential anti-AIDS effect. [Life Science Journal 7(2): 1 – 7] (ISSN: 1097 – 8135).

Key Words: Promoter of CXCR4, medicine screening, anti-AIDS agents.

I. Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) into target cell critically depends on two cell surface components, CD4 and a chemokine coreceptor. Usually the chemokine coreceptor refers to CXCR4 or CCR5 (CC Chemokine Receptor 5) [1-3]. The chemokine receptor CXCR4, a member of the superfamily of G-protein-coupled receptors (GPCRs) like CXCR1-CXCR6、CCR1-CCR11、XCR1 and XCR2 etc., is the first to be discovered HIV coreceptor [4], and mainly found on the surface of immune cells [5, 6]. The HIV-1 envelope (Env) consists of gp120 and gp41. gp120 contains the CD4 binding site and a hydrophobic fusion peptide directly involved in membrane fusion respectively. During the infection, CD4 binding induces conformational changes in gp120 that exposes the coreceptor binding determinants. The gp120 interaction with the coreceptor then induces a further conformational change in Env which results in insertion of the fusion peptide into the target cell membrane [3, 7, 8]. Then HIV-1 RNA integrates into the infected cell genome [9]. The study of anti-AIDS drugs targeting on HIV-1 membrane fusion and virus entry becomes a hotspot of research interests nowadays.

The coreceptors are important determinants of viral tropism and pathogenesis and are obvious targets for antiviral drug development. CCR5 mediates the entry of R5 viruses, previously called M-tropic, or non-syncytium-inducing viruses [2, 10-13], that is mainly isolated from patients in the early (asymptomatic) stage of HIV-infection. CXCR4 mediates entry of X4 viruses, previously called T-tropic, or syncytium-inducing viruses [1, 4], often emerge in HIV-infected persons in a

later stage of disease progression towards AIDS [5, 14]. The drugs target of interfering the CXCR4 expression is one of the main study in anti-AIDS/HIV drugs [15-20]. Thus, it may be possible to delay or prevent the progression of AIDS through decreasing the expression of CXCR4 by interaction of the activity of CXCR4 promoter. Traditional Chinese medicines are the peculiar drugs in China. Their pharmacology has a wide variety of different functions. Some of these traditional Chinese medicines, whether they could interact the CXCR4 promoter and cause CXCR4 expression decline, are worthy of further research.

The pGL4 Luciferase Reporter Vector is the next generation of reporter gene vectors optimized for expression in mammalian cells. It includes the synthetic firefly luc2 (Photinus pyralis) and Renilla hRluc (Renilla reniformis) genes, which have been codon optimized for more efficient expression in mammalian cells. We cloned the DNA fragment of CXCR4 promoter into pGL4 vector, then transfected the recombinant into Jurkat cells, and then select the transfected cell by G418. Thirteen kinds of traditional Chinese medicines were given to rats by gastric gavage once a day. 7 days later, the serum samples were separated. After being stimulated by the thirteen medicated serums, the luciferase activity inside the transfected Jurkat cells were analyzed in order to know the activity of CXCR4 promoter. Through analyzing the effects on the CXCR4 promoter transcriptions by the thirteen traditional Chinese medicines, we established an anti-HIV/AIDS drugs screening technique targeting on CXCR4 promoter.

TABLE I
DOSE OF TRADITIONAL CHINESE MEDICINE

Traditional Chinese Medicine	Crude Herbal Dose (g/ml)
Radix scutellariae (Huangqin)	0.63
Rhizoma coptidis (Huanglian)	0.35
Cortex phellodendri chinensis (Huangbo)	0.84
Flos lonicerae japonicae (Jinyinhua)	1.05
Fructus forsythiae (Lianqiao)	1.05
Herba lobeliae chinensis (Banbianlian)	1.05
Herba houttuyniae (Yuxingcao)	1.75
Folium isatidis (Daqingye)	1.05
Radix isatidis (Banlangen)	1.05
Ganoderma (Lingzhi)	0.84
Herba andrographis (Chuanxinlian)	0.63
Polyporus (Zhuling)	0.84
Radix et rhizoma glycyrrhizae (Gancao)	0.63

II. Materials and Methods

A. Materials

Traditional Chinese medicine of Radix scutellariae (Huangqin), Rhizoma coptidis (Huanglian), Cortex phellodendri chinensis (Huangbo), Flos lonicerae japonicae (Jinyinhua), Fructus forsythiae (Lianqiao), Herba lobeliae chinensis (Banbianlian), Herba houttuyniae (Yuxingcao), Folium isatidis (Daqingye), Radix isatidis (Banlangen), Ganoderma (Lingzhi), Herba andrographis (Chuanxinlian), Polyporus (Zhuling) and Radix et rhizoma glycyrrhizae (Gancao) were purchased from Zhangzhongjing Medicamentarius (Henan). All of them are recorded in the Chinese Pharmacopoeia. Active principles of Chinese herbs were extracted by decocting [21, 22]. Dose of traditional Chinese medicines (crude herbal dose in the decoction) are shown in Table I.

B. pGL4-CXCR4 Vector Construction

Primers with restriction enzyme sites of Kpn I and Nhe I (TaKaRa) were designed according to the DNA sequence of CXCR4 promoter: M1: 5'-CGG TAC CAA GCA CTA TTC GCG AAT TGG TTA C-3', M2: 5'-TGC TAG CGG TAA CCG CTG GTT CTC CAG A-3'. CXCR4 promoter fragment was obtained by PCR amplification from plasmid pUC-CXCR4. After digested by Kpn I and Nhe I, it was connected with pGL4 vector (Promega) using T4 ligase (QIAGEN). Positive recombinant vector pGL4-CXCR4 was identified through DNA sequencing and restriction enzyme digestion with Kpn I and Nhe I.

C. Cells Transfection and Selection

Jurkat cells were cultured in RPMI-1640 medium containing 100g/L fetal bovine serum at 37°C and 5% CO₂. The vector pGL4-CXCR4 and pGL4-CMV (contain the CMV promoter fragment) were transfected into Jurkat cells by LipofectamineTM2000 (Invitrogen) respectively. Then transfected cells were screened by G418 (Invitrogen) for 4 weeks. The concentration of G418 is 600µg/ml during the cell selection and 200µg/ml during the amplification culture of stable transfected cells.

D. Preparation Medicated Serum

Forty-four adult Wistar rats (180-220g), purchased from laboratory animal center of Zhengzhou University and used in the following experiments according to the guiding principles for the care and use of laboratory animals. Rats were randomly divided into fourteen groups. The rats of thirteen experimental groups were administered intragastrically with thirteen kinds of traditional Chinese medicines decoction respectively, for 3ml/rat (equivalent to 10 times of clinical equivalent dose for adult person of 60kg), once a day at 4pm-5pm for 7 days [23]. Blood was obtained by abdominal aorta puncture 24h after the last administration and the separated serum were termed as medicated serum. In one control group, rats were orally administrated normal saline in the same protocol; Their serum were used as control serum [24]. Both the medicated and control serum were inactivated by heating at 56°C for 30 min [25, 26], then filtered through 0.22 µm filter, and stored at -80°C until use. (The serum of same group were mixed for storage [27])

E. Stimulating Cells by Serum

The Jurkat cells stably expressing pGL4-CXCR4 were randomly distributed into fourteen groups: one is control serum group stimulated by control serum; the other thirteen experimental serum groups stimulated by thirteen medicated serum respectively. 2ml cell suspension at a density of 5×10^5 /ml was inoculated into 6-well plates and cultured in RPMI-1640 with thirteen 10% medicated serum and one 10% control serum respectively. There were 5 sub-wells in each group and incubated at 37°C in a humidified incubator with 5% CO₂. 24h later, the same culture medium was changed once again. Meanwhile, the untransfected Jurkat cell was served as blank control group.

F. Luciferase Activity Assay

After a further culture for 16h, the cells were collected (3×10^5 /well) and the luciferase activities of the reporter gene were measured by using E1500 luciferase assay kit (Promega) according to the manufacturer's protocol. Fluorescent value was detected by using GlomexTM20/20 luminometer (Promega). Each well was performed three times.

TABLE II
DETECTION OF LUCIFERASE ACTIVITY

Group	n	Fluorescent Value	P
Radix scutellariae (Huangqin) Group	15	899839.6 ± 20855.6	.389
Rhizoma coptidis (Huanglian) Group	15	865065 ± 46150.0	.908
Cortex phellodendri chinensis (Huangbo) Group	15	406175.2 ± 35146.2*	.000
Flos loniceræ japonicæ (Jinyinhua) Group	15	804546.4 ± 19439.3	.073
Fructus forsythiæ (Liangqiao) Group	15	930277.2 ± 25787.3	.089
Herba lobeliae chinensis (Banbianlian) Group	15	1146839 ± 106138.3*	.000
Herba houttuyniæ (Yuxingcao) Group	15	612186.8 ± 38890.9*	.000
Folium isatidis (Daqingye) Group	15	927398.6 ± 106458.0	.105
Radix isatidis (Banlangen) Group	15	850857.4 ± 81690.6	.607
Ganoderma (Lingzhi) Group	15	921582.0 ± 113271.1	.144
Herba andrographis (Chuanxinlian) Group	15	867962.6 ± 62413.1	.973
Polyporus (Zhuling) Group	15	843579.0 ± 87317.3	.473
Radix et rhizoma glycyrrhizæ (Gancao) Group	15	832654.2 ± 92261.2	.306
Control Group	15	869159.0 ± 62618.8	
Blank control Group	15	39088.4 ± 3867.9	

*There are significant difference between the control group and experimental group ($P < 0.05$)

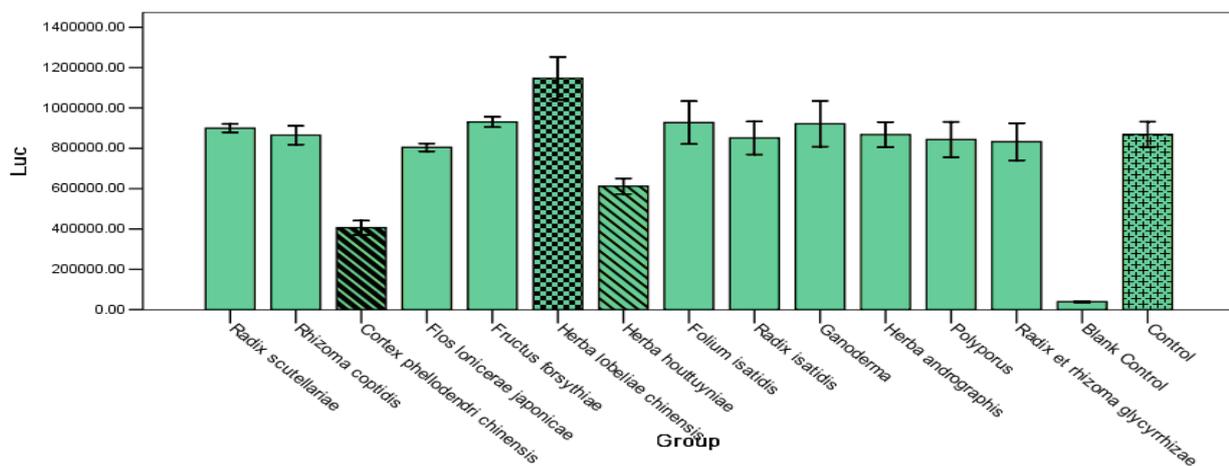


Fig. 3. Detection of the luciferase activity. There are significant difference between the control group and experimental group (Cortex phellodendri chinensis group, Herba lobeliae chinensis group and Herba houttuyniæ group). ($P < 0.05$)

G. Statistical Analysis

All data were analyzed with SPSS 13.0 Software and the results were expressed with means ± standard deviation. Data in groups were compared with single factor variance analysis (ANOVA). $P < 0.05$ showed significant difference.

H. Western Blot Analysis

Four groups of cells (Cortex phellodendri chinensis group, Herba lobeliae chinensis group, Herba houttuyniæ group and control group) were sonicated and purified protein were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to Western blot analysis, using a polyclonal antibody to CXCR4 as a primary antibody. Sheep anti-rabbit horseradish peroxidase-conjugated antibody was used as a secondary antibody. The bands were coloured with electrochemiluminescence method.

I. Analysis of Independent Control Group

Using the same method, the Jurkat cells stably expressing pGL4-CMV were distributed into four groups and stimulated by four kinds of medicated serum: Cortex phellodendri chinensis serum, Herba lobeliae chinensis serum, Herba houttuyniæ serum and control serum. Then the fluorescent value was detected and the data were analyzed.

III. Result

A. PCR Amplification Results of CXCR4 Promoter

CXCR4 promoter gene fragment was amplified by PCR from pUC-CXCR4. PCR products were detected by agarose gel electrophoresis. The detected outcome is that typical electrophoretic band is close to DNA marker 770bp. The outcome is consistent with the theory (777bp). See the captions for Fig. 1.

B. Identification of Recombinant Vector

The positive pGL4-CXCR4 plasmid was digested by Kpn I / Nhe I and then confirmed by their digestion. The result of agarose gel electrophoresis showed that the band is

TABLE III
THE LUCIFERASE ACTIVITY DETECTION (pGL4-CMV)

Group	n	Fluorescent Value	P
Cortex phellodendri chinensis (Huangbo) Group	15	899839.6 ± 20855.6	.843
Herba lobeliae chinensis (Banbianlian) Group	15	85166.5 ± 82304.6	.193
Herba houttuyniae (Yuxingcao) Group	15	841427.8 ± 58223.1	.110
Control Group	15	893616.6 ± 64088.2	

more or less around 770bp (Fig. 2). At the same time, insertion sequence in pGL4-CXCR4 was confirmed identical with the designed sequence of CXCR4 promoter by sequencing.

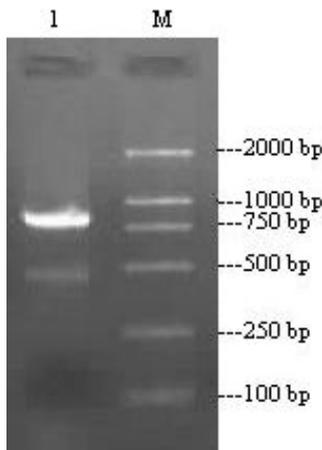


Fig. 1. Agarose gel electrophoresis of PCR products. 1: PCR products of CXCR4 promoter; M: DNA Marker DL2000

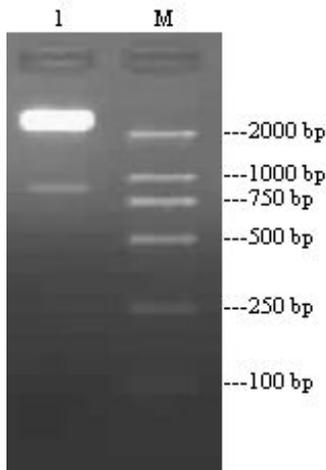


Fig. 2. Agarose gel electrophoresis of the recombinant plasmid after digestion with Kpn I / Nhe I. 1: digestion result of pGL4-CXCR4; M: DNA Marker DL2000

C. Result of Luciferase Activity Assay

Fluorescent value of each group cells is detected by Glomex™20/20 luminometer after these cells were stimulated by the medicated serum. All data of the detection were analyzed with SPSS 13.0 Software, and conducted with single factor variance analysis (ANOVA). Variance of any a totality is equal to another, P<0.05 was considered significant. The results of detections are

shown in Table II and Fig. 3. The test data shown that the fluorescent value of Herba lobeliae chinensis group was significantly increased when compared with the control group (P<0.05); the fluorescent value of Cortex phellodendri chinensis group and Herba houttuyniae group were significantly decreased when compared with that of the control group (P<0.05). These show that the luciferase activity in the Jurkat cells of Herba lobeliae chinensis group was significantly higher than those in the control, while the luciferase activity in the Jurkat cells of Cortex phellodendri chinensis group and Herba houttuyniae group were greatly decreased. Because the segment of CXCR4 promoter was inserted into pGL4 vector, we can know that the CXCR4 promoter activity should increase in Herba lobeliae chinensis group than that in the control; the CXCR4 promoter activity should decrease in Cortex phellodendri chinensis group and Herba houttuyniae group. All data were analyzed statistically and shown with bar chart (Fig. 3). From this bar chart, the fluorescent values of each group could be compared directly, and there were obvious difference from the control group between the Herba lobeliae chinensis group, Cortex phellodendri chinensis group and Herba houttuyniae group. In addition, according to Table II and Fig. 3, we can find that the fluorescent value of the control group was 20+ times than those in the blank control group, which suggests that the Jurkat cell was transfected stably with pGL4-CXCR4 and expressed the luciferase activity.

D. Western Blot for CXCR4 Protein

CXCR4 Western-blotting (Fig. 4) showed that protein level in Cortex phellodendri chinensis group and Herba houttuyniae group were higher than that in control group; protein level in Herba lobeliae chinensis group were lower than control group.

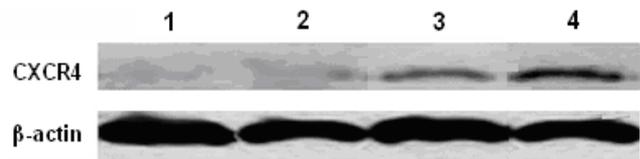


Fig. 4. The comparison of CXCR4 protein expression 1-4: Cortex phellodendri chinensis group, Herba houttuyniae group, Control group, Herba lobeliae chinensis group.

E. Result of Independent Control Group

The Jurkat cells stably expressing pGL4-CMV were stimulated by four kinds of serum, the luciferase activity in the cells showed no difference between control group and medicated serum group.(P>0.05) (Table III, Fig. 5)

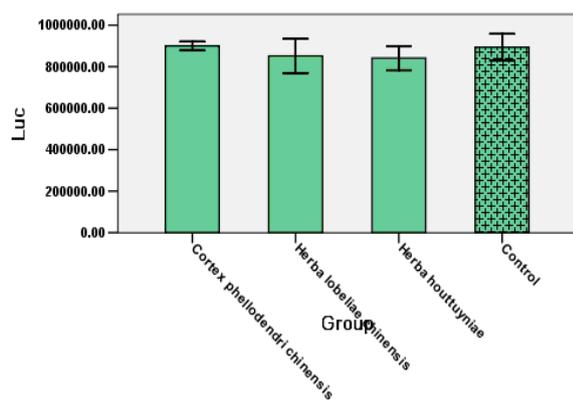


Fig. 5 The luciferase activity detection (pGL4-CMV)

IV. Discussion

Chemokines are small soluble proteins of about 70 amino acid residues and its molecular mass is 8 to 10 kD. They are natural ligands of the most known HIV coreceptor. CXCR4, a member of CXC chemokine receptor, is the principal coreceptor and mediates entry of the T-cell-tropic HIV-1 isolate which is involved in the onset of AIDS-defining symptoms [3, 28, 29]. Study in vivo and in vitro show that antagonism or function interference of CXCR4/CCR5 can have a marked effect on HIV (super-) infection at the entry step, thus inhibit HIV-1 replication. The purpose of this experiment was to establish a medicine screening system to investigate the effect of some medicines acting on CXCR4 promoter. These medicines have the efficacy of down-regulation CXCR4 expression, then play its roles of anti-AIDS.

Chinese herbs serum pharmacology method was proposed by Japanese scholar in 1984. It is an in vitro method of using medicated serum which was collected after the animal was given single Chinese herb or compound traditional Chinese medicine oral administration. It has become an important method on traditional Chinese medicine pharmacology research recently. This method avoids the interference of herb physicochemical properties in vitro study when the herb is used directly. It also reflects the pharmacologic actions of herb and its metabolites, and better fit the actual process in the living body environment [30]. If the crude preparation of traditional Chinese medicine adds directly into reaction system (e.g. cultured cells) the nonspecific physical and chemical factors (such as pH values, osmotic pressure, tannins and inorganic salts etc.) in the crude preparation will effects on cell growth, thus produce false positive results or false negative results. These false positive results & false negative results will affect the results' reliability and scientificity. But, the serum can add directly into reaction system and be a single & controllable influencing factor to evaluate the experimental results, if using serum pharmacology method. This serum contained the effective components of drugs after the body absorbs and metabolize these drugs. This kind of method can reflect drug effect and mechanism objectively and directly. Moreover, some drugs could affect the CXCR4 promoter not by their

original form but by metabolic form, or some drugs could affect the CXCR4 promoter directly but lose this ability after these drugs were absorbed. Metabolism and factors in the serum can influence these drugs effect. My above descriptions could be reflected accurately by the serum pharmacology method. So, the experimental results can be considered more reliability by this method [31]. Animals currently employed for the study are rats [27]. But, there are differences in absorption and metabolism of drugs between animals and human body. So, the proper drug dose is important to ensure the success of the experiment. Firstly, should consider that because serum amount should not be bigger and diluted many times the plasma concentration should be as big as possible. And secondly, should consider how the drugs could achieve their proper concentration of the best effectiveness. Some scholars reported that cytotoxicity of the serum will inhibit cell growth when using higher concentration drug serum. So they think that concentration of the drug serum should be 10% in vitro [32]. Other scholars think that the amount of added serum should not be over 20% by considering the serum tolerance of the cultured cells [32]. Therefore, the establishment of the amount of adding drug serum in vitro study should refer the reported literatures and we should select a proper amount according to the preliminary experiment results: avoiding the cytotoxicity of higher concentration of drug serum, and making the drug serum concentration close to the blood concentration in order to reflect the drugs holistic effect objectively. In this experiment, drug serum addition amount is 10% each well. To make drug concentration in vitro identical with those in vivo, dosage given to rats by oral administration is: commonly used clinical amount \times equivalent area coefficient of animal \times sera diluted of culture medium [33]. Drug feeding times was in accordance with routine usage of 7-10 days [23]. In addition, traditional Chinese medicine preparation has larger granules and easy interfered with food, therefore most of animals can absorb medicine effectively and quickly in the fasting state. The rat is a nocturnal animal and food intake at night. Giving drugs in the afternoon is also to obey this law [34]. For the treatment of experimental serum, we tend to inactivate the serum by heating at 56°C for 30 min [25, 26]. Inactivation not only can help to eliminate the interference of complement component activity, but also accord with the aseptic requirements of conventional cell culture [35]. Meanwhile, in order to control the authenticity and reliability of experimental result, when the experiments are arranged on the base of serum pharmacology method, we not only study the relationship between the serum adding amount and the effective drug concentration, but also set up control serum group to eliminate the interfering substances effect of serum itself on the study system [24, 35]. In this experiment, each group include three rats and the serum in the same group were collected for mixing use, which can also reduce individual difference in drug absorption among experimental animals [27].

There are two stages in the regulating of gene expression: transcriptional regulation and post-transcriptional

regulation. In this experiment we used reporter gene research method to investigate the transcriptional regulation of traditional Chinese medicine on CXCR4 promoter. If the screened medicines can up-regulated CXCR4 promoter activity they could induce the increase of CXCR4 gene transcription. CXCR4 gene transcription's increase will induce the CXCR4 protein expression level to increase, no change or decrease because of the existence of post-transcriptional regulation. Contrarily, if the screened medicines can down-regulate CXCR4 promoter activity and reduce the CXCR4 gene transcription, the diminution of CXCR4 protein expression will be finally affirmed. This is because the post-transcriptional can only down-regulate the gene transcription. Thus, the medicines' down-regulated effect on CXCR4 promoter is more reliable in our experiment. The western blot for CXCR4 protein coincide with the above-mentioned experiments.

At the same time, these three kinds of traditional Chinese medicines (Cortex phellodendri chinensis, Herba lobeliae chinensis, Herba houttuyniae) could not affect the activity of CMV promoter. These showed that the effects of these medicines for CXCR4 promoter were specific.

The experimental results show that Cortex phellodendri chinensis and Herba houttuyniae can down-regulate the activity of CXCR4 promoter. We speculate that Cortex phellodendri chinensis and Herba houttuyniae have potential anti-AIDS effect. Certainly, in order to confirm the preventative and therapeutical effect of these medicines on AIDS, we should also detect the expression of CXCR4 on the surface of CD4⁺ T cells, the absolute value of CD4⁺ T cells and the variation of viral loads in AIDS patients, etc. Also, because of (a) the serum were collected from the different individuals with the different physiological and pathological showing individual difference; (b) That serum containing drug stimulated cells by twice maybe cover up the drug's actual function; (c) The difference of absorption & metabolism between human and animal with the same drug and (d) the limit of some experimental conditions, this experimental method has some limitations. But, this experimental method is an effective screening system for anti-AIDS Chinese drugs, and is feasible for setup a rapid and high-throughput Chinese drugs screening program for CXCR4 promoter.

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