Research Strategies and Analysis of Traditional Chinese Medicine

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Abstract: Traditional Chinese medicines, beleaguered by researchers and scientists in the past for the ambiguity of mechanism in the clinical field, can be proven by many recent strategies and technologies for their efficacy. This article summarizes the role that Chinese medicine has recently played in the clinical field and also outlines some of the modern research methods used to study Chinese medicine. Phage display, c-fos expression, immunohistochemistry, and in-situ hybridization were summarized in the article. The advantages and shortcomings of specific bacteriophages and the methodology of Immuno & FISH were discussed in the article. A number of clinical examples were listed to provide the reference for the further analysis of TCMs.

1 INTRODUCTION

Traditional Chinese medicines (TCMs) are crucial components in clinical aspects and occupy an extremely important segment of the medical field. For some diseases, TCM has received a great deal of attention for its remarkable effectiveness. Chinese medicine has a long history of wide use in China for the prevention and treatment of various diseases by targeting and modulating multiple disease-related pathways with multiple effective components (Zimmermann 2007). Unlike new psychoactive substances (NPS) that would imperil mental [1]health and contain addictive chemicals, TCM has fewer side effects. Corydalis Yanhusuo (*yhs*) is widely used for the treatment of pain and inflammation. In dopamine D2 receptor knockout mice, its analgesic effects were attenuated in acute and neuropathic pain, but not in inflammatory pain assays. Thus, our results suggest that YHS is effective in reducing acute, inflammatory, and neuropathic pain without causing tolerance (Wang 2016). Compared to the original morphine, *yhs* which consisted of Corydalis and Angelica dahurica that are clinical commonly used classical prescriptions to alleviate pain (Wu 2015). These effects could only be achieved with psychotropic drugs such as morphine or opioids, which have a huge addictive potential. Compared to TCMs, chemical drugs act on a specific target of a single drug, but a pair of herbal medicines consists of many active ingredients. Different combinations of drugs can have different effects on physical therapy and herbal efficacy can change. For example, maoto (mahuang-tang), containing both ephedra herb and cinnamon bark, has a stronger diaphoretic effect and warming property than ephedra herb alone (Hayashi 2009). On the molecular level, the basic theory of TCM for the treatment or prevention of cancer is to restore the patient to a healthy state by altering multiple carcinogenic events. Wang et al. (Wang 2010) studied the oncogenic effects involving multiple abnormal genes/pathways, the use of TCM in cancer chemoprevention may be more advantageous than drugs that target a single molecule alone. In particular, resveratrol, curcumin, and berberine have all been evaluated in a number of clinical trials for the treatment of many types of cancer (McCubrey 2017). Fei (Fei 2018) studied 345 patients with locally advanced colon glands undergoing surgical resection divided equally into three groups with placebo, twice-daily intraperitoneal injections of 10 mg/kg of the herbal medicine catalase (treatment group), and twice-daily intravenous injections of 5 mg/kg of bevacizumab (control group) for a total of 12 weeks. Patient overall survival (OS), cancer-free survival (CFS) was significantly increased in the catalase-treated group. The clinical trial of the preliminary study concluded that treatment

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with the herb catalpol showed beneficial clinical outcomes, low cost, and no serious complications.

The study of herbal targets and their difficulties are due to the fact that the target can be a protein, DNA, or RNA that causes or contributes to disease. its validation consists of demonstrating that modulation of the target has a therapeutic effect. Assay development follows target validation and is an objective method for screening putative compounds to determine interactions and/or modifications with the target. Once the assay is established, the next step is to find compounds that can actively engage the target. From a library of potential compounds, a select number of leads are generated that demonstrate a relationship between chemical structure and target-based activity in a biochemical or cell-based assay (Improving and Accelerating Therapeutic Development for Nervous System Disorders: Workshop Summary 2014). The difficulties of multifaceted research have led to the achievement of this relatively difficult breakthrough in mechanism as well as target analysis in Traditional Chinese medicines. This article will focus on some ways that can effectively contribute to the research of TCMs and also exemplify the specific performance of TCMs in clinical and therapeutic aspects.

2 RECENT TECHNOLOGY

2.1 C-fos Expression

The c-fos gene is expressed in the central nervous system in neurons in response to various stimuli. The effects of chronic morphine treatment and withdrawal on c-fos mRNA in rat brains, especially in the identified striatal neurons (Bullitt 1990). Immunohistochemical methods may be used to identify the protein product, c-fos protein. As a result, after peripheral stimulation, c-fos expression may be used as a proxy for neuronal activation in the neuraxis (Bullitt 1990). Behavioral sensitization is believed to be implicated in opioid-seeking activities in humans, and is analogous to the intensification of drug craving following prolonged exposure (Cruz 2015). Learned connections between narcotics and the environment are believed to be encoded within complex patterns of sparsely spaced neurons called neuronal ensembles, which play an important role in addiction (Cruz 2015). Cellular imaging with the early gene c-fos and its protein product, in particular. Fos has been used to find sparsely scattered neurons that were highly stimulated during programmed drug activities like drug self-administration and context- and cue-induced drug finding reinstatement. Using three different experimental groups of saline, *yhs*, and morphine for comparison and measured by the combination of c-fos and immunohistochemistry, the effect of herbal medicine can be obtained. To date, no study has evaluated c-fos expression in vivo for YHS alone or the combination of YHS and morphine. Fos expression may help to understand more about the mechanism underlying YHS mode action. In addition, this study may help to understand new drugs or systems to target with opioids to block the negative side effects that come with opioids alone.

2.2 Immunohistochemistry And Fluorescent In-situ Hybridization (FISH)

Immunohistochemistry (IHC) binds fluorescent or colorable chemicals to antibodies and uses the immunological principle of specific binding reactions between antigen and antibody to detect the presence of target antigens in cells and tissues, which are then examined using light microscopy. This method allows observation not only of the amount of antigen present but also of where the antigen is located in brain sections. Fan (Fan 2014)summarized five milestones in the development and advancement of the IHC field include (1) the discovery of monoclonal antibodies that significantly improve diagnostic specificity; (2) a thermally induced antigen repair (AR) method that allows the most efficient IHC detection of formalin-fixed and paraffin-embedded surgical and cytological specimens; (3) a highly sensitive secondary detection system that can detect trace proteins on formalin-fixed and paraffin-embedded tissues with virtually no background staining; (4) an automated staining system that allows hundreds of IHC slides to be run in the same lab on the same day with reproducible and accurate results; (5) a digital pathology with imaging analysis digital pathology, allowing quality control using digital slides (full slide scanning) and further reducing turnaround time by making digital images (electronic slides) available through the website. Fluorescence in situ hybridization (FISH) is a molecular cytogenetics technique that uses fluorescent probes to bind to specific portions of nucleic acid sequences that have a high degree of sequence complementarity.

2.3 Combined Fluorescent In-Situ Hybridization (FISH) And IHC

Staining of opioid receptors will be carried out using fluorescence in-situ hybridization via RNA scope and standard IHC as described previously (Grabinski 2015). Twenty-micron brain parts will be incubated in a quenching solution containing H_2O_2 at room temperature for 45 minutes at the level of the hypothalamus. After that, the sections will be put on Fisherbrand Superfrost Plus Microscope Slides and baked in the hybridization oven overnight at 60 °C. Following the manufacturer's instructions, RNAscope in situ hybridization will be performed using the RNAscope Multiplex Fluorescent Kit (Grabinski 2015). The brain sections will be blocked for 1 hour at room temperature in a blocking buffer containing 10% natural donkey serum and 0.3 percent Triton X-100 (Phan 2020). The sections will incubate with a MOR antibody overnight. The sections will be cover-slipped using RNAscope DAPI, followed by Invitrogen ProLong Gold Antifade-Mountant. Images will then be captured using Leica Sp8 TCS confocal microscope (Phan 2020). Once again, ImageJ software will be used to analyze the number of c-fos containing cells. By combining the two approaches, the drug mechanism of *yhs* can be studied in depth.

2.4 Phage Display

Phage display, established by smith in 1985, is a unique genetic recombination technique to present polypeptide on the surface of filamentous bacteriophage. It is an effective screening method to detect the displayed protein and peptide. Foreign DNA fragments can be inserted into the gene (g3) of phage fd-tet. The phage, fd-tet, can be used as a cloning vector to produce cloned single-strained DNA. This single-stranded form can be packed inside the shell, indicating that phage particles (Smith 1985).

The basic principle of phage display technology is a gene encoding an exogenous polypeptide or protein is fused with a phage gene encoding a shell protein and eventually presented on the surface of the phage as a fusion protein.

Phages that have introduced various foreign genes are called phage libraries. The molecule to be studied is immobilized on the phage, and the phage library is used to screen for phage-carrying ligands that bind specifically to the molecule and isolate the phage in the library for display. Such processing ensures the biological function of the phage exogenous gene.

The advantages of phage display technology are (1) high efficiency of panning and the ability to select phages with high affinity. (2) The displayed peptides and proteins are internally linked to the phage, which facilitates the analysis of binding peptide and protein sequences. (3) All peptides and proteins encoded onto the exogenous genes are able to maintain their original spatial structure and biological activity. In recent years, this technology has shown its unique advantages in finding tumor-specific target molecules and targeted therapies. Phage display derivatives play an important role in the diagnosis and treatment of diseases and will be used in a wide range of applications in different medical technologies, including biosensing, monitoring, molecular imaging, gene therapy, vaccine development, and nanotechnology. Bacteriophage, a class of viruses that use bacteria as hosts, has a protein sshell and single-stranded or double-stranded DNA wrapped inside the shell. Based on the type of phages, phage displays can be classified as filamentous, T4, T7, and lambda (λ) phages. The structures of the phages are shown in Fig.1.

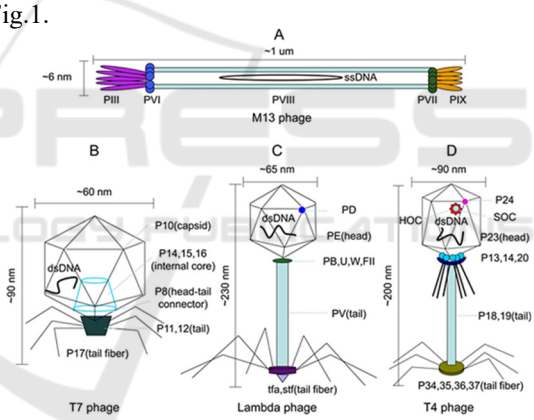

Figure 1: The structure of M13 (A), T7 (B), λ (C), T4 (D).

2.4.1 Filamentous M13 Phage

Based on Fig 2, the M13 phage genome is 6407 bp long and consists of structural proteins, replication proteins, and morphogenetic proteins encoding a total of 11 proteins. Among them, pVIII is the main coat protein of the phage display (Wezenbeek 1980). The M13 phage enters the cell through the flagellum on the surface of the host cell, therefore it only infects “male” E. coli with flagella. The spacer regions II/IV and VIII/III of the m13 phage genome can be used to insert exogenous DNA, and the majority of other regions are essential genes. The phage single-stranded DNA is encapsulated in a tubular structure consisting of approximately 2700-3000 molecules of

the major capsid protein pVIII. 424 amino acid residues of the pIII protein precursor can be inserted into the exogenous gene, and the protein encoded by the exogenous gene can be expressed as a fusion protein on the phage surface without disturbing the function of the phage (Shijie Huaren XiaoHua Zazhi 2003).

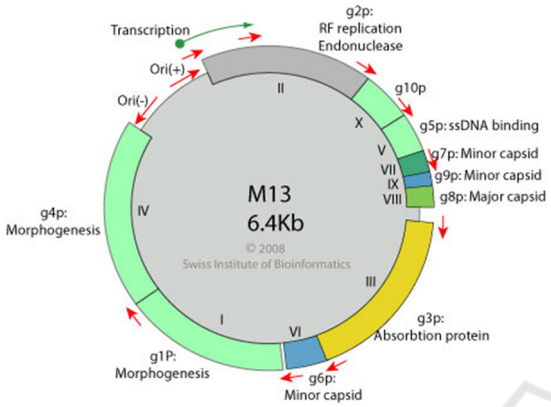

Figure 2: Single-stranded DNA of M13 bacteriophage.

2.4.2 M13KO7

The M13KO7 is a derivative of M13 bacteriophage with gene II of M13mp1. The replication origin of pl5A and the kanamycin resistance gene (K^+, kanr) of Tn 903 were inserted at the AvaI site (5825) of M13. With the pl5A origin, the phage was able to replicate independently of gIIp. This allows the phage to overcome the effects of interference and maintain sufficient genomic levels to express ssDNA in the presence of the phage to produce the desired protein (Vieira 1987). The pII protein introduced at M13KO7 is able to produce a stronger transcriptional effect with the viral replication start point, ensuring the preferential synthesis of the inserted gene.

2.4.3 T4 Phage

T4, the accessory proteins, HOC (highly antigenic outer capsid protein) and SOC (small outer capsid protein) connect to the capsid surface. SOC maintains the stability of the head and HOC is an elongated molecule protruding from the center of gp23* hexamer (The detailed structure of T4 bacteriophage is shown in Fig 3.). The T4 phage is characterized by its ability to fuse two exogenous polypeptides or proteins of completely different nature to the outer shell proteins SOC and HOC on the T4 capsid and display them directly on the surface of the T4 phage. It contains a large system capacity and achieves site display in vitro (Yap 2014). Although T4 phages can successfully display the structure, they have some limitations. Due to the restriction of SOC molecules trimerization with C-terminal interactions, they are not commonly used.

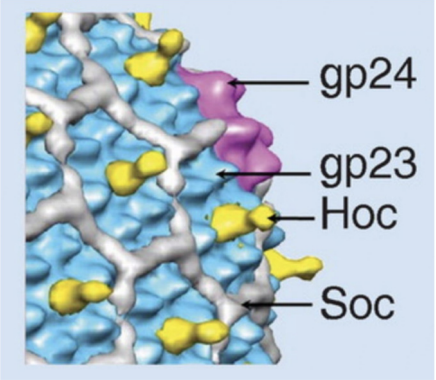

Figure 3: The structure of the T4 head in part.

2.4.4 T7 Phage

The double-stranded DNA of the T7 phage makes it more stable and less prone to genetic mutations during replication. The capsid of the T7 phage is mainly composed of gp10A and gp10B, whose main function is to protect the DNA inside. The multiple properties of the T7 phage make it a better research tool. The structure is shown (Fig. 4) can visually show that the outer shell plays a crucial role in protecting the DNA structure. Large protein fragments can be incorporated into coat proteins at low levels. Secondly, the coat shell consisting of g10A and g10B can effectively survive under harsh conditions such as high salinity, high pH value, and even denatured environments. Its growth rate can enable multiple rounds of selection in a single day. However, its drawbacks are also more obvious, because T7 phages are assembled in the cytoplasm and released by lysing bacteria, which leads to the use of T7 phages that are extremely destructive to the host cells (Tan 2016).

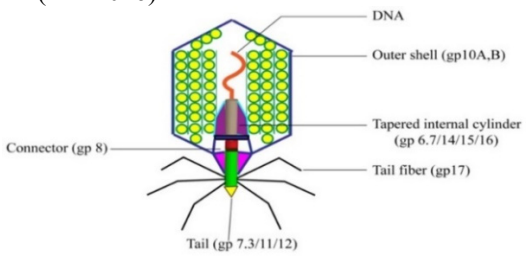

Figure 4: The structure of T7 phage bacteria.

2.4.5 Lambda Phage (λ)

Lambda is a mild E. coli phage whose lytic nature and

predominantly protein structure plays a significant role during phage display. During lysis, the cyclic DNA knows the synthesis of proteins required for viral replication, phage particle assembly, and cell lysis.

The advantage is that in the lysed state, the phage integrates itself into the chromosome of the host cell via lysogen and its lambda DNA resides in the host's genome without causing significant harm to the host. However, intracellular activity in the host cell is not easily monitored.

2.5 Current Use for Locating Targets

Phage presentation technology can be well combined with Chinese medicine into an effective target screening tool for drugs. It can present exogenous proteins and peptides on the phage surface and utilize the specific affinity interaction of exogenous proteins and peptides with the unknown to be screened. And the phages that fail to bind will be discarded. Afterward, the bound phages are eluted with reagents, the eluted phages are collected and infected with bacteria for amplification, and the resulting phages are then repeated for the screening process. In this way, after 3-5 rounds of "adsorption-elution-amplification" cycle screening, phages that specifically bind to the treated phage exogenous peptide can be found. After gene sequence analysis, the corresponding basic amino acid sequences, which bind specifically to the target molecule, are obtained based on the sequencing results. The proteins in the organism containing the above amino acid sequences are analyzed with the help of bioinformatics to obtain the corresponding target proteins in the organism (Feng 2021).

The advantage of this technique is that the phage is easy to amplify and select, and the protein or peptide obtained after screening can also be determined by determining the DNA sequence of the insert. Rodi (Rodi 1999) applied this method to screen a random library of phage display peptides and the binding of biotinylated derivatives of paclitaxel (Taxol). The binding of paclitaxel to the anti-apoptotic human protein Bcl-2 was confirmed by ELISA assay. Zhang (Zhang 2017) studied that the phage library of 15 peptide random sequences was used to screen for specific cellular targets of strychnine, interacting with cell-selective binding peptides to investigate the mechanism of antitumor activity of strychnine. Sun (Sun 2016) analyzed that the specificity and molecular interactions between the candidate binding protein Ubiquinol-cytochrome c reductase binding protein (UQCRB) and oxymatrine were investigated using the T7 phage technique. The interaction between the two demonstrated that UQCRB is a potential target for the treatment of chronic hepatitis B (CHB).

3 CONCLUSION

Nowadays, TCM has triggered the research of many scientists and there are many methods available to determine the targets and active ingredients of TCM. Each herbal medicine has its own composition and validation methods. In the paper, a summarization of the pros and cons of several common methods and use cases would provide references for further analysis. Considering the complexity and diversity of the components and targets of TCM, it is difficult for a single method to effectively analyze get an accurate determination. The combination and practice of multiple methods can better achieve the desired results and can also improve the efficiency and accuracy of the purpose.

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