Screening of candidated molecular markers in polymyositis using GenMAPP software

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Abstract: **Objective** To screen and identify candidated molecular markers in polymyositis (PM). **Methods** The differential gene expression profile in PM was constructed. Gene MicroArray Pathway Profiler (GenMAPP) software was used to analyze the changes of microassay gene expression profiles and to identify candidate molecular markers in PM. The results of differential gene expression profile were furthermore verified by real-time quantitative RT-PCR, and Western blot were performed to confirm the differential expressed gene. **Results** Compared with normal muscle tissues, we found 119 genes, including 8 expressed sequence tag (EST) genes with 3 folds differential gene expression among 17 000 genes in PM. A total of 64 genes and 47 genes were found up-regulated and down-regulated in PM, respectively. These genes were found to be associated with cell proliferation, signal transduction, cell cycle, gene transcription, and DNA damage and repair. RT-PCR and Western blot showed that the expression levels of *NF-κB*, *TNF-α*, and *IL-1α* genes were remarkably up-regulated in PM. **Conclusion** The development of PM might be regulated by multi-gene expression and *NF-κB*, *TNF-α*, and *IL-1α* genes might be candidate molecular markers of PM.

Key words: polymyositis; GenMAPP; gene expression; protein expression; molecular marker

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利用 GenMAPP 筛查多发性肌炎候选分子标志物

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[**摘要**] **目的**:筛查、鉴定多发性肌炎候选分子标志物。**方法**:构建多发性肌炎的差异基因表达谱。利用 GenMAPP 软件对多发性肌炎和正常肌肉组织基因微阵列表达谱结果进行分析,筛查其候选的分子靶标,随后运用 real-time quantitative RT-PCR 和蛋白印迹方法进行验证。**结果**:在 17 000 个基因中,与正常肌肉组织相比、在多发性肌炎中发生 3 倍以上差异表达的基因共有 119 个,其中 8 个为 EST。表达差异的已知基因共 111 个,64 个表达上调、47 个表达下调。这些基因分别与细胞增殖、信号转导、细胞周期调节、基因转录及 DNA 损伤修复等相关。经 real-time quantitative RT-PCR 和蛋白印迹验证发现 *NF-κB*,*TNF-α* 以及 *IL-1α* 等基因在多发性肌炎中表达明显上调。**结论**:多发性肌炎的发生发展存在多基因表达调控的改变,其中 NF-κB,TNF-α 以及 IL-1α 等是其候选的分子标志物。

[**关键词**] 多发性肌炎; 微阵列; 基因表达; 蛋白表达; 分子标记物

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Gene ontology is the corresponding notes for biological process of genes, cellular component, and molecular function. Harhay et al[1] pointed out that in the era of functional genomics, gene ontology provides important information for gene function study. Gene MicroArray Pathway Profiler (GenMAPP) is a new and effective tool for analyzing of microarray results, searching for relevant biological pathways and molecular targets[2-3]. Studies have pointed out that the organic combination of gene ontology and the MAPPFinder function of GenMAPP lead to quick understanding of biological function of genes[4]. Through the gene ontology, we can get more complete understanding of the information of differentially expressed genes in the entire microarray expression profiling, providing a meaningful clue for follow-up study of gene function and disease candidate molecular target selection and validation.

Polymyositis (PM) is inflammatory myopathies caused by autoimmune reaction, which account for about 70% of idiopathic inflammatory myopathies. PM has two peak of onset age, 5 ~ 14 years old children and 45 ~ 64 years old adults. The prevalence rate of PM in female population is 2 times of the male. Additionally, there were markedly racial differences in the frequency of PM. Some studies showed that the frequency of PM in African-Americans are 4 times of the Caucasian's and the lowest prevalence rate in Japanese adult[5-6]. The mainly clinical characteristics of PM are weakness, myalgia and proximal limb myatrophy and its pathological change is mononuclear cells (MNC) infiltration, however its molecular pathogenesis remains unknown[7-9]. Up to date, some researchers thought that the rapid isolation and identification of differentially expressed genes related to PM morbidity[10] are key techniques to explore molecular mechanisms of PM occurrence and screen its molecular markers. In this study, we used GenMAPP software to investigate the changes of microassay gene expression profiles and to identify candidate molecular markers in PM and normal muscle tissue. Our results will provide a new clue for further study of molecular mechanism of PM development.

1 MATERIALS AND METHODS

1.1 Tissue prepration

Ten PM and 10 normal tissue samples from outpatient clinics were collected in Xiangya Hospital, Central South University. PM diagnosis was based on the diagnostic criteria of Bohan. One PM patient for gene microarray analysis was from a male, 47 years old. His major clinical manifestations were weakness, upper more serious, severe biceps and proximal myatrophy. The main feature of pathological change in this patient was mononuclear cells infiltrating. All PM and normal control tissue samples were divided into 2 parts, one part was used to make pathological diagnosis (no infiltration of inflammatory cells were thought to be normal control tissues), another tissue was stored in RNAlater in −20 ℃ for the determinations of mRNA and protein.

1.2 Microarray membrane and main reagents

GALAXY™ (Model: HC10001) cDNA array film was prepared by Shanghai Zhongkekairui company[11]. The number of membrane probes was 17 000, including about 16 000 genes and about 1 000 expressed sequenced tag (EST), each point on the chip was repeated twice. Trizol™ reagent was Gibco-BRL product (Gaithersburg, MD, USA), isotope [α-^{33}P] dATP was purchased from Beijing Yahui company, mRNA isolation kit was purchased from Qiagen Inc. (Valencia, CA, USA), RT kit and labeling kit were purchased from Promega corporation (Madison, MI, USA). Mouse anti-human MMP-2 polyclonal antibody, mouse anti-human MMP-9 polyclonal antibody, sheep anti-mouse antibody, mouse anti-human β-actin monoclonal antibody were purchased from Santa Cruze (USA). SYBR Green I kit was from TAKARA company (Dalian, China). The ammonium persulfate, TEMED, Tris, and other ordinary reagents were purchased from Beijing Huamei company.

1.3 Extraction of total RNA and mRNA isolation[11]

Total RNA of PM biopsy tissue and normal muscle tissue was extracted according to the instruction of Trizol™. RNA was digested with RNase free DNase I. The quality of tatal RNA was controlled by of agarose gel electrophoresis. After that, mRNA reverse transcript, probe marking, hybridization, washing film and so on were done.

1.4 Image analysis[11-13]

DNA microarray contained a total of 17 000 genes, each gene has 2 points. FLA-3000A Plate signal scanner from FIJIFILM company was used to scan and analyze the data. Fluorescent image analyzer software was selected to input X-ray information to computer and Array Gauge software was used for analysis of gene expression abundance. Using housekeeping gene as internal standard, the results of two hybrid membrane were normalized. Gene expression intensity (density) were obtained from the average hybridization signals from two points of the same gene on the microarray membrane. Gene expression density ratio (ratio) > 3.0 or < 0.3333 was considered to be differential expressed gene.

1.5 GenMAPP 2.0 software analysis

The differential gene expression levels were analyzed by use of the GenMAPP 2.0 software[2]. The downloading, installing, and using instruction for GenMAPP 2.0 software refer to following website: http://www.GenMAPP.org.

1.6 RT-PCR validation of microarray results

Three most obvious up-regulated expression genes in PM tissue were selected to verified by RT-PCR. The primers were designed by the Primer 3 software. Primers were synthesised by Shanghai Boya Biological Limited Company. Primers sequences were as follows: *NF-κB* forward 5′-cctggatgactcttgggaaa-3′, reverse 5′-tcagccagctgtt tcatgtc-3′; *TNF-α* forward 5′-tccttcagacaccctcaacc-3′, reverse 5′-aggccccagtttgaattctt-3′; *IL-1α* forward 5′-aatgacgccctcaatcaaag-3′, reverse 5′-tgggtatctcaggcatctcc-3′; *GAPDH* forward 5′-gtcagtggtggacctgacct-3′, reverse 5′-aggggagattcagtgtggtg-3′. iQ5 fluorescence quantitative PCR cytometry was used to detect. Amplification conditions: 93 ℃ 2 minutes predegeneration, 93 ℃ 45 seconds, 55 ℃ 60 seconds, 10 cycles, 93 ℃ 30 seconds, 55 ℃ 45 seconds, 30 cycles. Blank control was set for each test. The specificity and reliability of PCR results were judged according to the melting curve and the agarose gel electrophoresis of PCR products. After the reaction, computer automatically generated the C_T value (threshold cycle values), the gene expression intensity was standardized according to the C_T value, house-keeping gene (*GAPDH*) and calculated by $2^{-\Delta\Delta CT}$[14].

1.7 Protein extraction and Western blot analysis

PM and normal control tissue samples after grinding were added protein extraction lysis buffer (50 mmol / L Tris-Cl pH8.0, 150 mmol / L NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/mL PMSF, 1 μg/mL Aprotinin, 1% Nonidet P240, 0.5% Na-deoxycholate) and cracked on ice for 20 ~ 30 minutes, then cell debris was removed after 13 000 r/min centrifuge and protein concentration of the supernatant was determined with BCA kit. Total protein (50 μg) was used for SDS-PAGE gel electrophoresis. The gel was electrically transferred to cellulose nitrate membrane, then the membrane was blocked by TBST containing 5% skim milk powder at room temperature for 2 hours, incubated by primary antibody overnight, and then incubated by the corresponding secondary antibody marked by horseradish peroxidase at room temperature for 2 hours. The signal was detected by enhanced chemiluminescence. a-tubulin was used as housekeeping gene and experiment repeated thrice.

1.8 Statistical analysis

All data are expressed as the mean ± SD. Differences between two groups were analyzed by independent samples *t* test using the SPSS software package (Version 13.0 for windows; SPSS, Chicago, Illinois, USA). $P < 0.05$ was considered statistically significant.

2 RESULTS

2.1 Differential gene expression profile in PM

Total RNA in PM and normal muscle tissue was extracted. The result showed that the extracted RNA had no gDNA pollution and the intact ribosomal RNA (28S and 18S bands). Fig. 1 showed the cDNA chip hybridization scanning map in PM and normal muscle tissue. Each dot on cDNA microarray map in Figure represented a gene or a EST. The density value of each gene or EST was determined by the hybrid imaging density of the point itself in the X-chip contrast with the array in the background compared to subtractive hybridization density. Therefore, it would be more reliable to reflect the relative expression level of the gene or EST in the tissue.

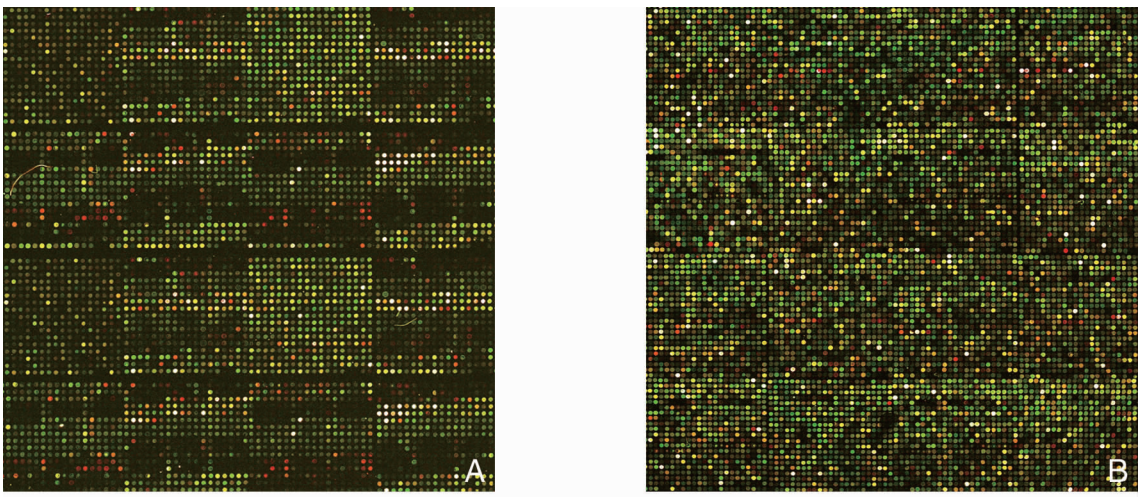

Fig. 1 cDNA chip hybridization scanning map. A: Polymyositis tissue; B: Normal control tissue.

2.2 Analysis of differential expression gene function

According to the average of the ratio of the same housekeeping gene on two membranes, normalized coefficient was caculated. All the data times normalized coefficient were the value of adjusted target gene signal. However, the point of genes whose signal strength values are less than 400 were discarded. The ratio value was greater than 3 or less than 0.333 in all target genes were calculated, which showed a larger differential gene expression in PM and normal control tissue. GenMAPP 2.0 software was used to analyze gene expression.

According to the above screening criteria, we found that there were 119 genes those differential expression ratio value were greater than 3 or less than 0.333 between PM tissue and normal controls tissue. Among these differential expression genes, there were 67 gene down-regulated in PM biopsy tissues and 52 gene expression up-regulated, other 8 differential genes belong to the newly cloned or not cloned unknown new EST. One hundred and eleven differential genes with known function had been gene ontologied in biological process, cellular component and molecular function. These genes could be classified into the following categories: (1) cell cycle regulation related genes; (2) structure protein genes related to cytoskeleton; (3) genes related to DNA damage repair; (4) nuclear pore complex signal transduction related genes; (5) telomere length regulation related genes; (6) genes participate in extracellular matrix signal regulation; (7) metabolism related genes; (8) immune associated genes (Tab. 1 ~ 2). We found that the expression levels of *NF-κB*, *TNF-α*, *IL-1α*, *Mcp-1*, *Hsp90*, *MMP-2*, *Fas*, *p53*, *Bcl-2*, and *HLA-IPM* genes were significantly up-regulated in the PM and the up-regulation multiples of these genes were 5.17,

5.05, 4.91, 4.85, 4.83, 4.79, 4.17, 3.90, 3.84, and 3.82, respectively. They participated in B cell receptor antigen processing and presentation, neurodegenerative disorders, and GnRH signal transduction pathways.

2.3 Expression of *NF-κB*, *TNF-α* and *IL-1α* mRNA

cDNA chip analysis showed that more than 10 genes such as *NF-κB*, *TNF-α*, *IL-1α*, *Mcp-1*, and *Hsp90* were significantly up-regulatied in PM tissue. In order to verify the chip results, we choosed the *NF-κB*, *TNF-α*, and *IL-1α* genes with most up-regulation multiples of gene expression in 10 normal muscle tissue and 10 PM tissue and their expression levels were quantitated by RT-PCR. Results showed that *NF-κB*, *TNF-α*, *IL-1α* genes were lowly expressed in normal muscle tissue and highly expressed in the PM muscle tissue, and their expression levels were 3.61, 2.53, 2.31 times of normal control group, respectively ($\Delta\Delta C_T$ calculation method: $\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ GAPDH})_{PM} - (C_{T,\ Target} - C_{T,\ GAPDH})_{NT}$; the multiples of differential gene expression in PM with normal contrast were calculated by $2^{-\Delta\Delta CT}$ (Tab. 3). The results of gene expressions determined by RT-PCR were basically coincided with the results detected by gene chip.

2.4 Western blot analysis of NF-κB, TNF-α and IL-1α protein expression

In order to determine the expression of NF-κB, TNF-α and IL-1α protein, we randomly selected five normal tissues named N2, N3, N5, N7, N10 and 5 PM patients tissues named T1, T4, T5, T6, T8 for Western blot. The positive bands were analyzed by Gelpro4 version of gel optical density analysis software and the IOD values were determined. The results showed that the IOD values of NF-κB and TNF-α proteins in PM patients were significantly higher that those in the normal control group (125.6 ± 10.4 vs. 52.3 ± 9.4, $P < 0.01$; 198.1 ± 13.5 vs. 77.8 ± 10.7, $P < 0.01$). Moreover, we found that the IOD value of IL-1α protein in PM patients was also markedly higher than that in the normal control group (287.2 ± 17.4 vs. 199.9 ± 13.2, $P < 0.05$) (Fig. 2). The results of Western blot were absolutely consistent with the results of RT-PCR.

Tab. 1 Biological process, cell composition, and molecular function of 52 genes lowly expressed in the PM

Classification	Number	Z score	*P* value
(A) Biological process			
cytoskeleton tissue and biogenesis	42	3.123	0.0140
Regulation of actin polymerization and/or depolymerization	26	2.783	0.0230
negative regulation of metabolism	31	2.534	0.0360
lipid catabolism	6	2.871	0.0220
biopolymer metabolism	39	2.570	0.0251
regulation of lipid metabolism	3	3.097	0.0273
actin filament-based process	7	2.778	0.0097
brain development	6	2.453	0.0325
(B) Cellular component			
cell	37	2.345	0.0327
intrinsic to plasma membrane	12	3.013	0.0274
hemoglobin complex	23	2.924	0.0110
integral to plasma membrane	5	2.661	0.0402
clathrin vesicle coat	29	3.086	0.0309
clathrin coat of trans-Golgi network vesicle	31	2.945	0.0256
cell projection	12	3.034	0.0313
clathrin coat	7	-2.922	0.0098

续 表

Classification	Number	Z score	*P* value
(C) Molecular function			
protein serine/threonine kinase activity	26	3.434	0.0295
transcriptional repressor activity	11	2.534	0.0200
carboxylic ester hydrolase activity	4	−2.152	0.0396
calcium ion binding	17	3.479	0.0019
kinase activity	22	2.460	0.0194
ligase activity, forming carbon-nitrogen bonds	7	-2.691	0.0377
ion binding	14	-2.797	0.0118
phosphotransferase activity, alcohol group as acceptor	20	3.046	0.0092

P values and Z scores were obtained through analysis of MAPPFinder in GenMAPP software. $P<0.05$ was considered as statistical difference.

Tab. 2 Biological process, cell composition, and molecular function of 67 genes highly expressed in the PM

Classification	Number	Z scores	*P* values
(A) Biological process			
protein ubiquitination	57	3.327	0.0280
sensory perception of light	38	2.722	0.0149
actin cytoskeleton tissue and biogenesis	46	2.343	0.0356
regulation of cell tissue and biogenesis	31	3.401	0.0198
visual perception	19	2.714	0.0328
metal ion transport	21	2.610	0.0203
potassium ion transport	13	3.721	0.0012
membrane lipid metabolism	17	2.561	0.0205
(B) Cellular component			
nucleus	46	3.601	0.0199
chromosome	61	2.703	0.0411
cell	34	2.924	0.0318
clathrin coat of trans-Golgi network vesicle	25	3.015	0.0202
trans-Golgi network transport vesicle	16	2.413	0.0390
nucleosome	17	2.805	0.0170
cytoplasm	25	2.614	0.0300
spindle pole	7	2.194	0.0450
(C) Molecular function			
nucleotide binding	55	2.267	0.0230
phospholipase activity	31	3.323	0.0221
transferase activity	29	2.456	0.0180
cation channel activity	37	2.614	0.0250
kinase activity	12	2.523	0.0190
protein kinase activity	30	2.456	0.0180
guanyl nucleotide binding	43	2.122	0.0460
transferase activity, transferring phosphorus-containing groups	9	2.456	0.0180
transferase activity	15	2.173	0.0340

P values and Z scores were obtained through analysis of MAPPFinder in GenMAPP software. $P<0.05$ was considered as statistical difference.

Tab. 3 RT-PCR validation of *NF-κB*, *TNF-α* and *IL-1α* genes ($\bar{x} \pm s$)

Gene	Sample	GAPDH C_T	Gene C_T	ΔC_T	$\Delta\Delta C_T$	Multiples of expression differences
NF-κB	N	21.43 ± 0.99	24.81 ± 1.06	3.38		
	T	20.01 ± 0.87	21.54 ± 0.87	1.53	1.85	3.61
TNF-α	N	21.41 ± 0.77	23.82 ± 0.93	2.41		
	T	19.96 ± 0.94	21.03 ± 0.78	1.07	1.34	2.53
IL-1α	N	20.53 ± 0.80	23.25 ± 0.65	2.72		
	T	19.60 ± 1.02	21.11 ± 0.90	1.51	1.21	2.31

N: Normal control tissue; T: Polymyositis tissue. ΔC_T = Gene C_T-GAPDH C_T, $\Delta\Delta C_T = \Delta C_{TN} - \Delta C_{TT}$, multiples of expression differences = $2^{-\Delta\Delta CT}$.

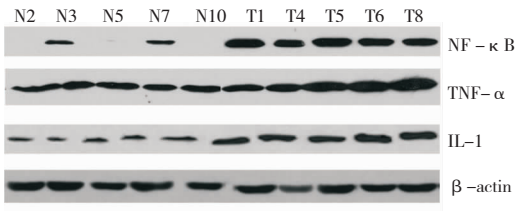

Fig. 2 Western blot results of NF-κB, TNF-α and IL-1α protein. Lane N2, N3, N5, N7, and N10 represented the normal control tissues, respectively. Lane T1, T4, T5, T6, and T8 represented the PM patients tissues, respectively.

3 DISCUSSION

Human genome draft has been completed ahead of schedule, now the era of functional genomics research has came. Human is facing on more difficult task that is to examine the function of genome, which means not only to understand the genome sequence, but also to master the temporal and spatial information of genome structure. In quite a long time, our major work will be focusing on revealing the information of all genes on transcription[15].

Gene chip is a high-throughput molecular biological technology and rapidly developed since the mid 90s. Gene chip or microarray technology can rapidly and accurately analyzes gene expression with its unparalleled amount of information and high throughput[16-19]. The level of gene expression reflects function information in different environment, cell type, stage of cell growth and the state of cell. It is very important to draw the expression map of all genes for the study of gene function. With advantages of large-scale, high-throughput and paralleled processing as of expression profile chip, a space-time map reflecting all the genes expression under all the conditions including the normal, abnormal and controlled could be drawn. So the one-dimensional human genetic map becomes multi-dimensional. The bioinformatics, comparison and statistical analysis of each gene expression could explore the relevance of different genes expression and find the function of unknown genes and unknown function of known genes as well as provide clues to further explore the function of these genes and screen disease molecular markers[18-19].

The aim of molecular biology research of PM was to find important individual genes and understand their structure and function, and gene expression status in cells. Rapid isolation and identification of PM related differential expressed genes are the foundation of studying the molecular mechanism of its occurrence and progression. In this study, we used the gene chip combined with GenMAPP analysis to screen candidated molecular targets in PM. We found that there were 119 genes in PM with more than 3 times of differential gene expression compared with normal control. Among these genes, the expression levels of 67 genes and 52 genes in PM biopsy tissues were down-regulated and up-regulated, respectively. Moreover, our results showed that these differential expression genes were involved in cell cycle regulation, DNA damage repair, nuclear pore complex signal transduction, regulation of te-

lomere length, regulation of extracellular matrix signal, and metabolic and immune processes. The gene expression of *NF-κB*, *TNF-α*, *IL-1α*, *Mcp-1*, and *Hsp90* were significantly up-regulated, which suggested that these genes might play an important role in the progression of PM. Nagaraju et al[20] found that TNF-α-positive $CD8^+$ lymphocytes infiltrated in non-necrotic muscle fibers of PM, which implied that TNF-α and lymphotoxin might be involved in muscle injury and inflammatory response in PM. It was well known that TNF-α could activate NF-κB, and NF-κB in turn reduced MyoD, which is a myogenic transcription factor necessary for the formation of new muscle fibers. TNF-α together with IFN-γ could damage muscle fibers severely. Therefore, TNF-α had dual role in preventing the formation of new muscle fibers and damaged muscle fibers resulting in severe loss of muscle volume[20]. Li et al[21] found that high TGF-β expression existed in the blood vessels, inflammatory cells and extracellular matrix in PM. Expression of nuclear NF-κB was significantly increased in muscle fibers and infiltrating mononuclear cells in PM patients. In the inflammatory response, TGF-β had dual role, at first it induced pro-inflammatory response and it then produced the immunosuppressive effects. Additionally, IL-1α played an important role in the occurrence and progression of PM[22]. Therefore, we thought that the *NF-κB*, *TNF-α*, and *IL-1α* genes played an important role in the occurrence and progression of PM and they are probably good candidated molecular markers as the diagnosis and threatment of PM. In the present study, we successfully found that there were significantly differential expression of *NF-κB*, *TNF-α* and *IL-1α* genes at the transcriptional and protein levels between the PM patients samples and normal control tissue samples. Our results not only confirmed the reliability of the gene chip technique, but also prompted their possibility as molecular markers in the diagnosis of PM.

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(Edited by GUO Zheng)

征 稿 启 事

《中南大学学报(医学版)》原名《湖南医科大学学报》,创刊于1958年,为教育部主管、中南大学主办的高级医药卫生类综合性学术期刊。该刊已被美国医学文献分析和联机检索系统(MEDLINE)及其《医学索引》(*IM*),荷兰《医学文摘》(*EM*),美国《化学文摘》(*CA*),俄罗斯《文摘杂志》(*AJ*, *VINITI*),中国科学引文数据库(核心库)(CSCD)等国内外多家重要数据库和权威文摘期刊收录;是中文核心期刊、中国科技论文统计源期刊及中国期刊方阵"双效"期刊;多次被国家和省部级新闻和出版部门评为优秀科技期刊,2008年被评为"第2届中国高校精品科技期刊"、"中国精品科技期刊"和湖南省"十佳科技期刊"。

本刊辟有述评、论著、综述、病例报告、科研快报等栏目。现面向全国高等医药院校、医药卫生系统和海外相关研究机构的作者征集优秀的中、英文稿件,尤其欢迎国家攻关项目、重点科研项目及重大基金资助课题的有关研究论文,本刊已设置相关研究报道专栏,并为优质稿件开设"绿色通道"。本刊稿约见本刊网站:Http://xbyx.xysm.net。

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