·Basic Research·

Comparative proteomic approach in differentiating multicentric occurrence and intrahepatic metastasis in multinodular hepatocellular carcinomas

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[Abstract] Background and Objective: Multinodular hepatocellular carcinoma (HCC) might originate from multicentric occurrence (MO) or intrahepatic metastasis (IM).We screened differentially expressed proteins between these two groups using comparative proteomic approach in order to find out proteins characteristic for clonal origin. **Methods:** Total protein extracted was separated by two-dimensional gel electrophoresis (2-DE). Comparative analyses of the 2-DE protein patterns between the two groups were carried out using computerized imaging techniques. Proteins exhibiting significant alternations were subsequently isolated and identified by mass spectrometry. **Results:** A total 1025±52 and 900±98 spots were detected in the protein profile in IM and MO, respectively. Twenty-five protein spots were statistically different at expression levels between the two groups. Twenty of them were identified by MALDI-TOF-MS and bioinformatics. **Conclusions:** The protein profile of MO HCC tissues is different from that in IM HCC tissues. The twenty differentially expressed proteins might play a key role in the carcinogenesis and progression of multinodular HCC. These newly identified proteins might be potential and valuable biomarkers for identifying the multinodular HCC of clonal origin.

Key words: Intrahepatic metastasis, multicentric occurrence, multinodular hepatocellular carcinoma, proteomics

Hepatocellular carcino ma (HCC) is a common malignant tumor around the world. Among all HCCs, multinodular HCC accounts for 12%–19%.[1] The origin of multinodular HCC can be either intrahepatic metastasis (IM) from a single primary tumor, or multicentric occurrence (MO) in an individual nodule.[2] Comparative proteomics research strategy is to study the differential expression profile of proteins. Through comparing all proteins expressed in tissues/cells under different pathophysiological conditions or differential protein molecules among sub-cellular domains, it is possible to screen for molecular markers and therapeutic targets of clinical application values, thereby exerting a far-reaching impact on the diagnosis, treatment and prevention of cancers.

In this study, using two-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) technology, we analyzed the proteome expression profile of multinodular HCC tissues from both IM group and MO group so as to identify differential protein molecules that play important roles in the clonal origin of multinodular HCC, and to provide a foundation for screening protein markers for distinguishing the origins of HCC and establishing a diagnostic model .

Material and Methods

Tissue samples

Ten specimens of multinodular HCC tissues were collected

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from the patients who underwent tumor excision surgery in the First Affiliated Hospital of Guangxi Medical University between 2004 and 2007. Diagnosis was confirmed by postoperative pathological examinations. None of the patients had received any chemotherapy before the surgery and informed consent was obtained from each patient before sample collection.

Analysis of clonal origins of multinodular HCC

Cancer nodules derived from the same cell clone had matched gene expression profiles and were classified as oligocentric origin, while cancer nodules with different cell clonal origins usually had totally or partially non-matched gene expression profiles and were classified as multicentric origin.[3,4] Five parameters were used to distinguish the cell clonal origins of multinodular HCC, including immunohistochemical analysis of P53 and AFP proteins, gene mutation analysis of P53 exon7 and BCL-10, and mitochondrial DNA (mtDNA) D-Loop sequence analysis. If all 5 parameters in each cancer nodule were identical, the multinodular HCC was determined as of oligocentric origin, that is, the IM. If the 5 parameters in each cancer nodule were different or partially different, the multinodular HCC was determined as of multicentric origin, that is, the MO.

As a result, five of the 10 cases were identified as IM, while the other 5 were identified as MO. The 5 patients in MO group were men, aged 29–64 years, with a median age of 44 years, and had a total of 10 nodules, while the 5 patients in IM group had 12 nodules, and there were four men and one woman, aged 30–62 years, with a median age of 41 years.

Extraction of total tissue protein

In MO group, 0.1 g tissue was cut off from each sample, washed by normal saline, mixed, weighted and transferred into a pre-cooled mortar. Tissues were ground into powder in continuous supply of liquid nitrogen and powder was transferred into 2 mL EP tubes. Lysis buffer 400 μL per 50 mg tissue (30–80 mg) was added to extract the total protein, oscillated in ice bath for 1 h, centrifuged at 12000 × *g*, 4℃ for 60 min, and supernatant was collected to determine the protein concentration by Bradford assay and stored at -80℃ as aliquot. The same procedure was used to extract the total protein from samples in IM group.

Analysis of 2-DE and images

2-DE was performed following the manual of Amersham Biosciences and the protein loading amount was 400 μg. The first dimension was isoelectric focusing on an IPG phor horizontal electrophoresis apparatus, with a total sample loading volume of 250 μL. Isoelectric focusing was started after 12 h hydration. Voltage was raised to 8000 V, and the focusing was terminated when cumulative voltage reached 53 kV·h. After focusing, gel strip was balanced for 15 min in buffer containing Dithiothreitol (1%) and buffer containing iodoacetamide (2.5%), transferred onto the top of a 12.5% polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) electrophoresis at 15℃ and continued until bromophenol blue reached the gel bottom. Gel was stained by double-methenamine silver staining (analysis gel) and Coomassie brilliant blue (preparation gel). Images were scanned and spot detection and matching analysis were performed using ImageMaster 2D Platinum6.0 software (Amersham Biosciences Corporation). Proteins with ⩾ 2-fold differential expression and appearing in ⩾ 50% of the gels were selected for mass spectrometry.

Protein identification and database search

Differential protein spots were cut out from the preparation gel, de-stained twice in buffer, dehydrated twice by acetonitrile, and added with 12.5 ng/μL trypsin and kept at 37℃ overnight. Polypeptide extract buffer was added and incubated for 20 min, and the supernatant was removed and repeated once. Resulting polypeptide mixture was dried in nitrogen, added 0.7 μL matrix, sufficiently mixed, loaded on MALDI plate and read on ABI 4700 TOF/TOF mass spectrometer for peptide mass fingerprinting and tandem mass spectrometric identification. Mass spectrometric data was input to NCBI non-redundant database and searched using MASCOT engine.

Results

Analysis of protein expression profile in multinodular HCC tissues

Total proteins from mixed tissue samples in both IM and MO groups were subjected to 2-DE for 3 times to ensure the repeatability of the experiment. Three 2D gel maps were obtained for each group. Expression profiles of the two sets of proteins were similar, that is, proteins were mostly concentrated in a region of pI 4-8 (Figure 1). Using Image Master 2D Platinum6.0 software to divide gel into two categories for spots identification analysis, 1025 ± 52 protein spots were detected in IM group (n = 3), while 900 ± 98 protein spots were detected in MO group (n = 3). 2-DE gels with good quality and more spots were selected as reference gel and matching analysis was carried out in the two groups. Matched spots between IM group and MO group were 680 ± 42 and the inter-group match rate was 65.6%. Twenty-five protein spots with more than 2 folds of differential expression and covering more than 50% of gels were screened out.

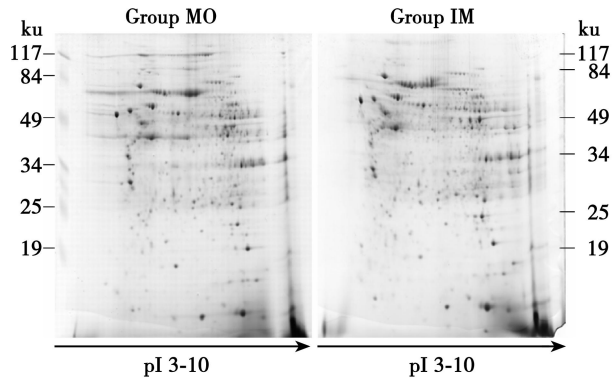

Figure 1 2-DE silver staining of group MO and group IM

Group MO, multicentric occurrence of multinodular hepatocellular carcinoma; Group IM, intrahepatic metastasis of multinodular hepatocellular carcinoma.

Mass spectrometric identification of differential protein spots

The 25 protein spots with relatively high differential expression were removed from the corresponding Coomassie brilliant blue stained preparation gel for MALDI-TOF-MS mass spectrometric analysis. Twenty proteins were identified, including lamin A/C isomer 2, (protein score ⩾ 60, Tables 1 and 2). Ten of these 20 proteins were up-regulated (Figure 2) and the other 10 were down-regulated in oligocentric HCC tissues (Figure 3). To ensure the accuracy when cutting spots, each protein spot was cut out from two gels to identify enzyme digestion and consistent mass spectrometric results were obtained. The peptide mass and tandem fingerprint spectrums of No. 5823 spot are shown in Figure 4.

Table 1 Proteins increasedly expressed in intrahepatic metastases identified by MALDI-TOF-MS.

No.	Protein	Database ID	Score	MW	PI	Coverage (%)
5580[b]	Lamin A/C isoform 2	gi|5031875	544	65095.6	6.4	67
5686	Protein disulfide isomerase	gi|860986	334	56643.7	6.1	48
6114	78 kDa glucose-regulated protein precursor (GRP 78)	gi|2506545	98	72377.5	5.07	11
6097	Carbonic anhydrase I	gi|4502517	370	28852.4	6.59	44
5823	Vimentin	gi|57471646	215	49623.1	5.19	38
5579[b]	Lamin A/C isoform 2	gi|5031875	445	65095.6	6.4	59
5792	Calreticulin precursor variant	gi|62897681	88	46890.1	4.3	19
4489	[a]Chain A, human serum albumin mutant R218p	gi|31615331	64	66369.9	5.62	18
4394	Alpha enolase[a]	gi|2661039	127	36285.6	6.53	47
4123	Chaperonin (HSP60)[a]	gi|306890	292	60986.4	5.7	28
4015	Glutamate dehydrogenase 1 variant[a]	gi|62897195	111	61301.2	8.05	3

[a]Specific spots in intrahepatic metastasis group.
[b]5579/5580 spots are identified as a same protein.

Table 2 Proteins decreasedly expressed in intrahepatic metastases identified by MALDI-TOF-MS

No.	Protein	Database ID	Score	MW	PI	Coverage (%)
5998	Glyoxylate reductase/hydroxypyruvate reductase	gi|6912396	103	35645.7	7.01	38
5981	Aging-associated gene 9 protein	gi|54303910	97	36026.4	8.57	22
6088	Ketohexokinase	gi|558216	491	25212.8	5.99	61
6339	Chain A, X-ray crystal structure of human galectin-1	gi|42542977	60	14583.2	5.34	26
5564[b]	Phosphoenolpyruvate carboxykinase 2	gi|12655193	534	70653.6	7.57	43
5563[b]	Phosphoenolpyruvate carboxykinase 2	gi|12655193	270	70653.6	7.57	27
6017	Tropomyosin	gi|339956	250	30362.4	4.64	39
5420	Aconitase 1	gi|8659555	390	98336.6	6.23	29
5915	Chain A, crystal structure of mutant macrophage capping protein (Cap G)	gi|33357110	177	38557.5	5.42	16
6134	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor[a]	gi|12707570	138	31351.1	8.34	15
5704	Keratin type Ⅱ[a]	gi|908805	84	60030.3	8.09	19

[a]Specific spots in multicentric occurrence group.
[b]5563/5564 spots are identified as a same protein.

Discussion

There are obvious differences between IM and MO HCCs in their treatment option and prognosis.[5,6] It is of important significance to distinguish IM and MO HCCs for pre-treatment evaluation, choice of treatment and prediction of survival. Currently, there are certain limitations in the molecular biologic techniques in identification of IM and MO. For example, mutation of P53 has a low expression rate in HCC.[7] Although mtDNA D-Loop region mutations may occur at any stage of HCC, screening of all cancer nodules fails to detect mutated cases, so determination of cell clonal origins of each nodule is relatively difficult. In this study, we used 2-DE plus mass spectrometry to analyze the protein expression profile of multinodular HCC from both IM group and MO group. We found differentially expressed proteins, including heat shock protein 60 (HSP60) and vimentin, which has provided a new clue to distinguish between IM and MO multinodular HCCs in aspect of proteomics and laid a foundation to search for more sensitive protein markers. However, some

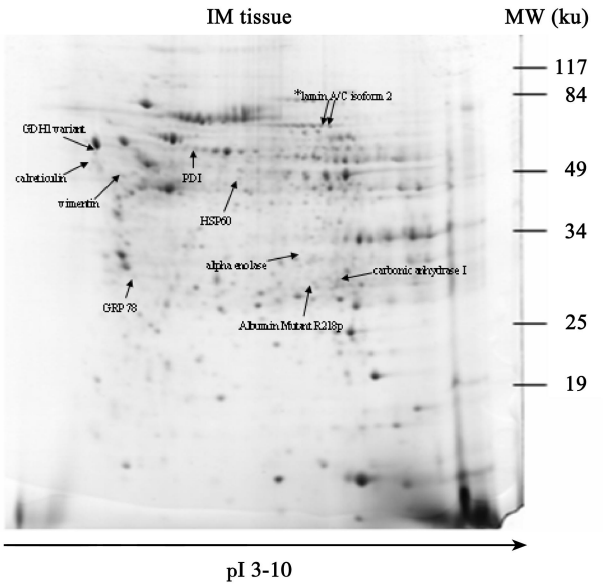

Figure 2 Protein spots increasedly expressed in intrahepatic metastases identified by MALDI-TOF-MS

Proteins detected by 2-D gel electrophoresis followed by silver staining in intrahepatic metastasis group (group IM) is presented; *5579/5580 spots are identified as a same protein.

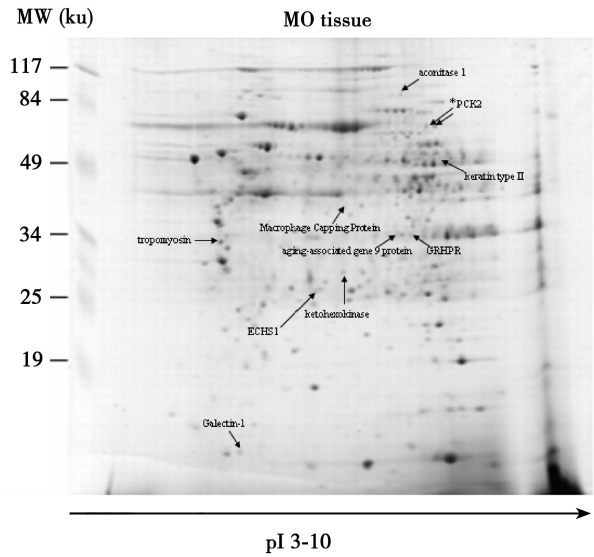

Figure 3 Protein spots decreasedly expressed in intrahepatic metastases identified by MALDI-TOF-MS

Proteins detected by 2-D gel electrophoresis followed by silver staining in multicentric occurrence group (Group MO) is presented; *5563/5564 spots are identified as a same protein.

factors of 2-DE such as limited sample number, sample processing and staining can interfere with the repeatability of experiment. Therefore, these differential proteins in the IM and MO HCC groups identified in our initial screening needed to be verified by further studies.

We applied 2-DE plus MALDI-TOF-MS and identified 20 differentially expressed proteins in the two groups. Nearly half of these differential proteins were enzymes involved in metabolism and biotransformation, and others included proteins related to cell signal transduction, cell mobility regulation, immune regulation and apoptosis modulation. Among the differentially expressed proteins, expression changes of some proteins in HCCs were novel among similar studies, such as glyoxylate reductase/hydroxy-pyruvate reductase, aging-related gene 9 protein and human serum albumin mutant R218p. So their roles in the cancerogenesis, invasion and metastasis of HCC need to be further studied.

In the 20 identified proteins,9 were involved in metabolism and biotransformation, but these enzymes or proteins did not directly participate in occurrence and development of tumors, suggesting that expression changes of these proteins were compensatory in the process of cancerogenesis in order to accommodate the high metabolic needs of uncontrolled growing tumor cells. α-enolase was also known as pyruvate phosphate synthase, which is an enzyme in the glycolysis pathway to catalyze formation of high-energy phosphate compounds from 2-phosphoglycerate. Takashima *et al.*[8] found that α-enolase expression was significantly increased in hepatitis C virus (HCV)-associated HCC tissues than in the corresponding non-tumor liver tissues, and its expression increased with dedifferentiation degree of tumors, that is, the expression level in poorly differentiated HCC was significantly higher than in well-differentiated HCC. Western blot analysis and immunohistochemistry confirmed that expression of this protein was positively correlated with tumor size and its venous invasion. In addition, Chen *et al.*[9] found that expression of α-enolase in HCC tissues was nearly upregulated by 3 folds that in paracancerous tissues. In this study, α-enolase was a specific protein expression spot in the IM group. We believe that the reason might be that during tumor invasion and metastasis, cells grew fast and aerobic oxidation reduced, so energy supply was dependent on glycolysis. Enoyl-CoA hydratase (ECHS) is also known as L-3-hydroxyacyl CoA hydratase, which is one of the key enzymes in mitochondrial and peroxisomal fatty acid β oxidation pathway and abundantly expresses in normal liver tissues. Reduced expression of ECHS was observed in HCCs.[10] Another study showed that events causing reduced function in mitochondrial oxidative phosphorylation could promote proliferation of transformed cells or tumor cells in oxidized tissues. Thus, the reduction in respiratory enzyme complexes was significantly correlated with the rapid growth and invasion of tumor cells.[11] In this study, mitochondrial ECHS 1 expressed in MO group, but not in the IM group, which supports the above-mentioned results.

HSP70 and HSP60 are two important heat shock protein families, with a high degree of conservation across species. The main functions of HSP60 in the body include stress, immune regulation, anti-apoptosis, signal transduction and so on. It is reported that HSP60 could bind to integrin α3β1 expressed in breast cancer cell membrane and increase the activity of integrin α3β1. Since the proliferation and invasion of tumor cells all

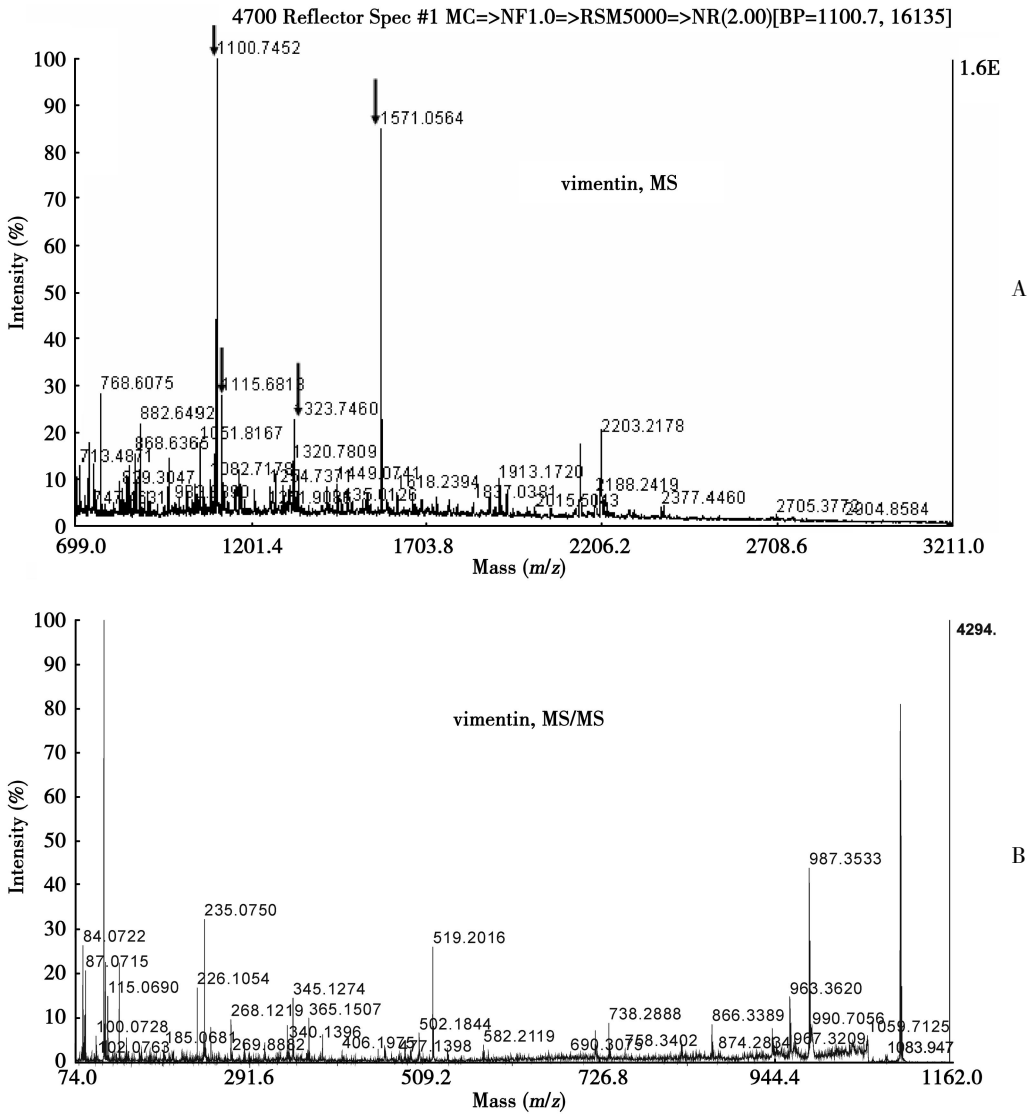

Figure 4 MALDI-TOF-MS result for spot 5823 (vimentin)

A, peptide MS map for spot 5823; B, tandem MS map for peptide 1100.74.

depend on the activity of integrin α3β1, it was believed that HSP60 played an important role in the metastasis of breast cancer cells.[12] Kuramitsu *et al.*[13] found that HSP60 was upregulated in HCV-related HCC tumor tissues. In this study, the expression of chaperonin (HSP60) was upregulated more in IM group than in MO group, supporting the above findings. Glucose regulated protein (GRP78) is also known as the immunoglobulin heavy chain binding protein (Bip), which is highly homologous to HSP70 family and is considered as one of the HSP70 family members. Luk *et al.*[14] reported that GRP78 expression was upregulated in HCC tissues, and was correlated with venous invasion of the tumor. They believed that increased chaperon protein expression in tumor cells was related to cell protection function, as well as proliferation and metastasis of tumors. Lim *et al.*[15] found that expression of GRP78 was gradually increased along with progress of hepatic B virus (HBV)-related HCC, and expression of GRP78 was correlated with the vascular invasion and intrahepatic metastasis of the tumor. Su *et al.*[16] found that specific down-regulation of GRP78 in vitro could inhibit the invasion and metastasis of HCC. In this study, the 78 kDa GRP 78 was upregulated more significantly in the IM group compared with the MO group. Protein disulfide isomerase (PDI) is a stress-related protein, as well as an important member of the endoplasmic reticulum molecular chaperone family. As an enzyme, PDI catalyzes the formation of disulfide bonds within protein molecules, and it also plays an important role as a molecular chaperone in the processes of protein translation and post-translational transport. Teramoto *et al.*[17] found that the expression of protein disulfide isomerase-associated 3 (PDIA3) was correlated with HCC and alpha-fetoprotein (AFP) level. In this study, expression level of PDI was higher in the IM group than in the MO group, suggesting that their expression levels were different at different stages of HCC.

Vimentin is one of the intermediate filament proteins, which

mainly expresses in mesenchyme-derived cells and some undifferentiated cells. However, it could also be abnormally expressed in some epithelium-derived cells, especially epithelium-derived tumor cells.[18] Hu *et al.*[19] applied cDNA microarray and immunohistochemical methods and found that vimentin was overexpressed in HCC cells and tissues and it was closely related to the metastasis of HCCs. YE *et al.*[20] used proteomics technology and found that vimentin protein expression was upregulated in HCC cells with high metastatic potential. In this study, vimentin expression was upregulated more markedly in the IM group as compared to MO group. Tropomyosin (TM) is a cytoskeleton protein, which is involved in processes such as cell movement, morphogenesis and actin filament regulation. YE *et al.* 20 reported that expression of TM in HCC cell M2H7402 of high metastatic potential was higher than in the parent cells. However, some other studies showed that expression of TMβ chain in HCV-related HCC was decreased.[13] Studies have shown that expression of TM2 was higher in early stage bladder cancer tissues than in advanced bladder cancer.[21] Sibley *et al.*[22] suggested that in high proportion of superficial transitional cell carcinoma (TCC), there were mutations in activated fibroblast growth factor receptor-3 (FGFR-3). Activation of this receptor was mediated by mitogen-activated protein kinase signaling pathway (MEK-ERK), and therefore it is possible that TM expression was down-regulated in these tumors. These results suggest that TM is associated with tumorigenesis and metastasis, and its action may be related to cell specificity. In this study, the expression of TM was down-regulated more markedly in the IM group than in the MO group, and the mechanism remains to be further studied.

In summary, we believe that application of proteomics strategy to screen for differential protein expression profiles of multinodular HCCs of different cell clonal origin could identify proteome changes of tumor cells under influence of multiple factors, and search for key protein molecules directly involved in the pathogenesis, progress, invasion and metastasis of tumors. These protein molecular markers that otherwise would be difficult to be identified using traditional methods might be molecules playing key roles in the pathogenesis, progress, invasion and metastasis of HCC. Further verification of expression and functional analysis would help clarify the pathophysiologic mechanism of multinodular HCCs of different cell clonal originsin order to identify molecular markers that could be used for early clinical diagnosis, efficacy determination, prognosis assessment and targeted treatment of HCCs.

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