

Fig. 4. (A) A photomicrograph of an HCC with adjacent non-cancerous cirrhotic liver stained with anti-SREBF1 antibodies. (B) Representative photomicrographs of SREBF1-negative-, SREBF1-low-, and SREBF1-high-HCC tissues stained with anti-SREBF1 antibodies. (C) SREBF1 gene expression by real-time RT-PCR according to protein expression status assessed by IHC. SREBF1 was highly expressed in SREBF1-high HCC ($P = 0.03$). (D) SREBF1 expression and cell proliferation in HCC. PCNA indexes in SREBF1-high HCC were higher than those in SREBF1-low/negative HCC with statistical significance ($P < 0.001$). (E) Kaplan–Meier plots of 54 HCC patients analyzed by immunohistochemistry. The differences between SREBF1-high and -low/negative HCC were analyzed by log-rank test.

developed in the liver of HCV core transgenic mice [42], strongly support our data. Although further studies using large numbers of HCC tissues may be required,

these data suggest that the lipogenic gene activation seems to be mediated, at least in part, by SREBF1 expression in HCC.

Table 2
Univariate Cox regression analysis of survival relative to *SREBF1* protein expression and clinicopathological parameters.

Variables (n)	HR (95% CI)	P-value
<i>SREBF1</i> and mortality (n = 54)		
Tumor size		
<3 cm (n = 37)	1	
≥3 cm (n = 17)	2.2 (0.6–8.3)	0.2
pTNM stage		
I, II (n = 45)	1	
III, IV (n = 9)	2.0 (0.4–9.4)	0.4
Serum AFP		
<20 ng/ml (n = 35)	1	
≥20 ng/ml (n = 19)	1.5 (0.4–5.4)	0.5
<i>SREBF1</i>		
Low (n = 34)	1	
High (n = 20)	3.7 (1.0–13.7)	0.05

Because the majority of our HCC patients analyzed had Child–Pugh class A scores and about 70% had tumors less than 3 cm in diameter, all were expected to have a good prognosis. Indeed, patient survival in this cohort was not segregated by tumor size or pTNM stage (Table 2). Although the sample size was relatively small, we found that enhanced expression of *SREBF1* was a prognostic factor for mortality in HCC possibly due to the highly proliferative nature. Activation of lipogenesis pathways, as shown by overexpression of *FASN*, has been found to correlate with high mortality in breast, prostate, and lung cancer [43], suggesting that activation of lipogenesis may be a fundamental characteristic of cancer with poor prognosis. Thus, *SREBF1* expression may be a good biomarker for HCC classification, a finding that should be validated in a large scale cohort. Because deactivation of the lipogenesis pathway by inhibition of *SREBF1* gene expression could inhibit HCC cell growth *in vitro*, *SREBF1* may be a good target for pharmaceutical intervention in these tumors.

In conclusion, our genome-wide gene expression profiling analyses found that the lipogenesis pathway was activated in a subset of HCC. *SREBF1*, which activates the lipogenesis pathway, may be a good biomarker for HCC prognosis and may be a good target for therapeutic intervention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.07.036.

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Application of Serial Analysis of Gene Expression in Cancer Research

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Abstract: It is now widely believed that tumors originate from normal cells as a result of accumulated genetic/epigenetic changes. These alterations affect the signaling pathways at transcriptional and post-transcriptional level that drive cells into uncontrolled cell division, growth, and migration. Recent advancement of molecular technologies have yielded comprehensive gene expression profiling techniques that have successfully provided candidate diagnostic and prognostic markers in human cancers. Serial Analysis of Gene Expression (SAGE) is a technology to facilitate the measurement of mRNA transcripts of normal and malignant tissues in a non-biased and highly accurate and quantitative manner. SAGE produces a comprehensive gene expression portrait without *a priori* gene sequence information, leading to the identification of novel transcripts potentially involved in the pathogenesis of human cancer. In this review, we provide a brief outline of SAGE to underscore the advantages of the method relative to the other gene expression profiling approaches in cancer research. We also summarize the progression of recent gene expression profiling studies and discuss the current topics of SAGE analysis in cancer research for the development of novel therapeutic interventions.

Keywords: SAGE, gene expression profiling, transcriptome, pathway analysis, biomarker, prognosis, metastasis, chemo sensitivity.

1. INTRODUCTION

Tumors originally develop from normal cells that gain the ability to grow aberrantly and metastasize to the distant organs. These malignant transformations are considered to be induced by the accumulation of multiple genetic/epigenetic changes [1, 2]. Although a classical genetic strategy such as gene knockout has been a powerful technique to explore genetic diseases with the gain or loss of a single gene, this strategy is less effective to obtain mechanistic insights of diseases affected by multiple genes such as cancer [3]. Complete sequences of the 3×10^9 base-pair human genome (as a result of the Human Genome Project (HGP)) have provided the basic framework of the genome-wide study in human diseases [4, 5]. Furthermore, genomic sequencing studies of various cancers have been reported or currently underway to reveal the genetic alterations on a genome-wide scale (The Cancer Genome Atlas; <http://cancergenome.nih.gov/>, The Cancer Genome Project; <http://www.sanger.ac.uk/genetics/CGP/>) [6, 7]. Comprehension of these framework studies will shed new lights on the genetic traits how cancer cells evolved from normal cells.

The central dogma is defined as the flow of genetic information from DNA to messenger RNA and then to protein, and the genetic/genomic alterations are considered to affect the cellular signaling pathway at transcriptional and post-transcriptional level. It is expected that the cataloging of tumor specific changes compared with normal counterparts

will provide mechanistic insights into the process of carcinogenesis and potential tumor markers [8]. In the past, gene expression was investigated by the simple imaging limited to gene-by-gene approaches such as Northern blotting and reverse transcription-polymerase chain reaction (RT-PCR). Over the past decade, several methods have been developed to allow comparative studies of gene (and protein) expression between normal and cancer cells at genome-wide scale upon requests in the post-HGP era [3]. In particular, two main technologies that allow the genome-wide detection of differences in gene expression, microarray and serial analysis of gene expression (SAGE), have been used to analyze gene expression in cancer research [9]. These technologies have successfully provided the candidate gene sets used for the early detection or prognosis prediction of cancer [10-13]. Furthermore, these technologies have provided the candidate signaling pathways activated and therefore can be molecularly targeted for elimination of tumors [14-16]. In this review, we provide examples of SAGE application for discovering tumor markers and subclasses for stratification of tumors with distinct clinical outcomes. We also discuss the future direction on the gene expression profiling approaches to better understand the cancer biology.

2. SAGE AND OTHER GENE EXPRESSION PROFILING TECHNOLOGIES

In early 1990s, Craig Venter and his group pioneered the novel approach named expressed sequence tags (ESTs), which was the first transcriptomic approach for characterization of a tissue [17-19]. ESTs are randomly selected clones sequenced from cDNA libraries at large scale. Each cDNA library is constructed from poly-A RNA derived from a tis-

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sue, and thus the library represents genes expressed in the original population. An EST consists of approximately 300 base pairs of DNA as a single run of a sequence that is sufficiently long to establish the identity of the expressed gene. Importantly, ESTs have provided novel genes as well as new members of gene families that are expressed in a tissue. The analysis of EST data revealed fundamental characteristics of gene expression such as which genes are abundantly expressed and which genes are differentially expressed in each tissue. Development of high-throughput sequencing technologies has enabled the acceleration of ESTs collection since then, and comprehensive samples of human ESTs have been compiled in a public domain: dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/>). More than eight million human ESTs sequences are currently available.

Although ESTs collection have provided huge amounts of gene expression information in human tissues including various tumors, deposited sequence information was derived from various institutes/laboratories with different methods to obtain cDNA libraries and their sequence data. Strong bioinformatics supports were required to quantitatively compare the gene expression changes between tissues in a non-biased manner using public ESTs database. To overcome these limitations of cataloging ESTs, Victor Velculescu reported a novel elegant gene expression profiling approach to effectively collect short EST sequences in 1995, called SAGE [20]. The novelty of SAGE technique is based on two points: 1) collection of a short sequence called a SAGE tag from a defined mRNA position that is enough to identify the genes, and 2) concatenation of SAGE tags to increase the efficiencies of sequence-based short ESTs collection. Development of SAGE has enabled to analyze tens of thousands transcripts expressed in a tissue in a highly quantitative and qualitative manner by one laboratory, and the results can be easily comparable among any SAGE libraries reported by others. More than 400 SAGE libraries derived from human tissues are currently available in a public domain GEO (<http://www.ncbi.nlm.nih.gov/geo/>).

Around the same time, Patrick Brown and David Botstein's group as well as Affymetrix team independently pioneered the innovative technologies called cDNA microarrays and GeneChip arrays, respectively, to measure the expression of genes simultaneously in a tissue [21-23]. Although the methods to immobilize the probes on glass slides as well as to label dyes in samples are different from each other, they share the basic concept to enable broad-scale gene expression profiling based on the sample-probe hybridization [24, 25]. The major advantage of using array-based technologies compared to SAGE is the high-throughput performance to obtain gene expression data of large numbers of samples. On the other hand, the disadvantages of array-based gene expression profiling are difficulties to compare data in different platforms and to obtain absolute quantity of mRNA expressed in a tissue. Additionally, whereas array-based profiling technologies are closed format and can only provide expression data of known genes, SAGE is an open format and can be utilized to discover novel genes or novel splice variants expressed in a tissue. Thus, SAGE and array-based technologies have both advantages and disadvantages, and the appropriate method should be chosen to fit the objectives

in each experiment. Besides, recent studies implicated the utility of combination of SAGE and DNA array technologies for target identification and validation studies to take advantages of both methods [26-31]. The outlines of brief SAGE and microarray protocol for cancer target identification are depicted in Fig. (1).

The gene signatures or biomarkers can be explored to discriminate or predict different clinical outcomes such as prognosis and chemotherapeutic sensitivities by pattern discovery and pattern prediction [9]. However, large-scale gene expression profiling data, especially these generated by SAGE, often provide false-positive candidates even after the rigorous statistical selection. Therefore, the results should be validated using different platforms such as real-time RT-PCR and/or tissue microarrays [32]. Furthermore, it is better to evaluate the significance of the identified gene signatures or biomarkers using an independent dataset, because the first data set used for the identification of biomarkers may not be representative of the diversity of patients for whom biomarkers are in broad clinical use [33].

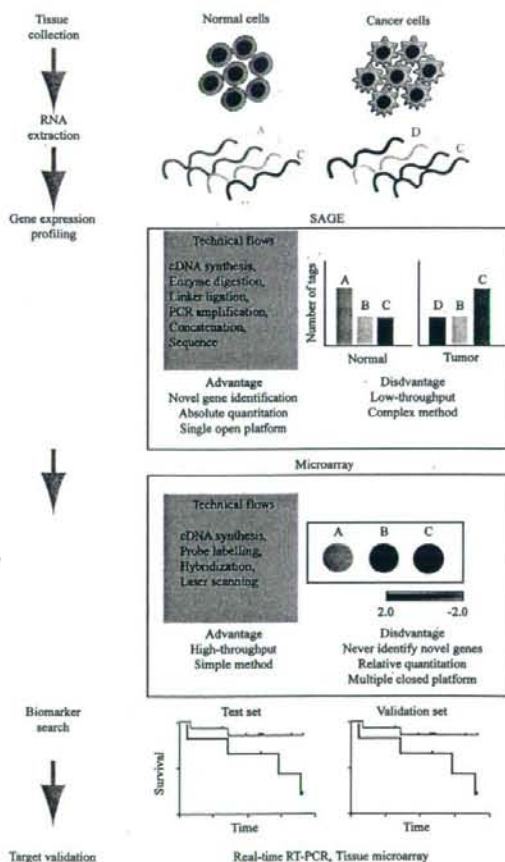


Fig. (1). The outlines of SAGE and microarray protocol for cancer target identification.

3. APPLICATION OF SAGE FOR CANCER TARGET/Biomarker IDENTIFICATION

3.1. Differential Gene Expression Between Normal and Tumor Tissues

SAGE has been used for the analysis of various tumors to decipher the pathogenesis of cancer development by comparing the gene expression patterns with those of corresponding normal tissues [34-36]. The first application of SAGE for comparison of normal and tumor tissues was reported in 1997, analyzing the expression profiles of colon and pancreatic tumors [37]. Although the authors showed the extensive similarity between normal and cancer tissues of the same organ, they discovered approximately 500 transcripts differentially expressed between normal and cancer cells, including many novel genes. Since then, various types of tumors have been analyzed by SAGE including lung, breast, ovarian, brain, gastric, and liver cancers [35, 38-44]. These results have been compiled in the SAGE map (<http://www.ncbi.nlm.nih.gov/SAGE/>) as a part of the Cancer Genome Anatomy Project (CGAP) and GEO database, and the expression of genes in each tissue type can be visualized in SAGE Anatomic Viewer as Digital Northern Results (<http://cgap.nci.nih.gov/SAGE/AnatomicViewer>).

These large amounts of data of various cancers have elucidated the principles of gene expression patterns commonly observed in tumors, and major findings have been well documented in recent reviews [32, 45]. The fundamental characteristics of cancer transcriptome are reemphasized here: 1) tumors share major gene expression traits with normal tissues that they originate from, 2) tumors increase the expression of genes associated with cell proliferation signaling, and 3) tumors decrease the expression of genes associated with the function of differentiated normal cells. These characteristics of cancer transcriptome evoke the dedifferentiated cellular phenotypes of cancer cells with an ability to grow rapidly. On the other hand, SAGE has also provided the candidate genes altered in specific type of tumors such as colon, breast, ovarian, brain, liver, and pancreatic cancers [46]. For example, *GPC3*, which encodes a heparan sulfate proteoglycan Glypican 3, was initially considered as a down-regulated gene in various cancers including mesothelioma, ovarian, and breast cancers [47]. However, up-regulation of *GPC3* was identified in hepatocellular carcinoma (HCC) by the differential display and SAGE [44, 48]. More recent studies have demonstrated up-regulation of Glypican 3 in majority of HCC at the protein level and suggested its utility for early detection of HCC [49-51]. Although the usefulness of biomarkers identified by gene expression profiling (e.g. *GPC3* in liver cancer) still remains elusive, further evaluation may reveal the utility of identified markers for early detection of specific type of cancers [44, 52, 53].

3.2. Elucidation of Signaling Pathway Targets in Cancer

The study of colon carcinogenesis in familial adenomatous polyposis patients suggested the stepwise accumulation of genetic alterations involved in various signaling pathways at distinct histopathological stages, implicating that about 4-6 genetic events are required for malignant transformation of human epithelial cells [54, 55]. Recently, Bert Vogelstein

and his group have explored genetic changes accumulated in cancers by sequencing approximately 13,000 protein coding genes distributed on whole genome [6, 7]. Interestingly, the analyses suggested that there are ~80 DNA mutations that alter amino acids in cancer cells. They performed statistical analyses to distinguish gene mutations that affect the tumorigenicity (driver mutations) from those do not (passenger mutations), and suggested that <15 DNA mutations were likely to be responsible for driving the initiation, progression, or maintenance of the tumor. Although not all mutations are likely to drive the initiation and promotion of the tumor, the number of DNA mutations in developed tumors seems to be higher than previously expected. The authors have discovered a number of candidate cancer genes mutated in breast and colorectal cancers (referred as candidate cancer genes; *CAN*-genes). Strikingly, the analysis of 11 colorectal cancer genomes has ascertained the mutations of oncogenes and tumor suppressor genes that are known to play a pivotal role in the pathogenesis of colorectal cancer, including *APC*, *KRAS*, *EPHA3*, *TGFBR2*, *TP53*, and *SMAD4*. Furthermore, they have demonstrated that many genetic alterations detected by cancer genome sequencing could be integral to smaller number of cell signaling pathways, suggesting the potential involvement of these novel mutated genes in the known characterized pathways.

SAGE is an ideal method to detect all alteration of transcriptome induced by the activation of certain signaling pathways, and has been utilized to identify key target genes in many signaling molecules [56]. The first example of SAGE application for target identification was reported in 1997, using colorectal cancer cell lines to discover p53 targets [57]. Remarkably, the analysis had provided 7 genes previously unrelated to the p53 signaling such as *PIG3*, supporting the strength of the method to provide novel insights into p53 signaling. SAGE has also been utilized to identify the targets of other signaling pathways such as adenomatous polyposis coli (*APC*), c-Myc, transforming growth factor- β (*TGF- β*), Tek/Tie2, and Von Hippel-Lindau (*VHL*)/hypoxia [14-16, 58-62]. A part of these studies are summarized in recent reviews [56], and most of these genes are now recognized as representative targets of each signaling pathway, including peroxisome proliferator-activated receptor (*PPAR*)- δ and c-Myc in Wnt/ β -catenin signaling pathway [14, 15]. Thus, SAGE is a potentially powerful tool to identify key targets of signaling pathways in cancer. The application of SAGE may provide important information to build a connection map of signaling pathways activated by *CAN*-genes.

3.3. Identification of Biomarkers Associated with Prognosis

Investigating Oncogenes/Tumor Suppressor Genes

In general, prognosis of a cancer patient is determined by the nature of tumor cells in terms of proliferation and metastasis. Consistently, analyses of microarray datasets of various tumors revealed the correlation of genes involved in cell growth and proliferation with poor outcomes [63]. Initiation and promotion of tumors are supposed to be dictated by the activation of oncogenes as well as inactivation of tumor suppressor genes, and most of these genes are known to regulate

cell cycle and proliferation [1, 2]. Therefore, investigation of oncogenes/tumor suppressor genes expression may enable prognostic stratification of cancer patients, such as reported in p53 mutations [64, 65]. Since the majority of SAGE data are publicly available, it is feasible to have a glimpse of the expression patterns of any genes of interest in various tumors at once. A recent study utilized the public SAGE dataset for identification of oncogenes associated with poor outcomes in ovarian cancer [66]. The authors investigated the expression of the gene families BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger) known to be mutated in B cell lymphoma using SAGE libraries, and identified the activation of *NAC1* in ovarian tumors. They further confirmed the overexpression of *NAC1* in other tumors such as pancreatic, liver, prostate, kidney, and breast cancers using SAGE libraries publicly available. Notably, overexpression of *NAC1* correlates with recurrent ovarian cancers, suggesting the utility of *NAC1* for stratification of ovarian cancers with poor prognosis.

Exploring Signaling Pathways

Considering the fact that most of gene products seem to work in a form of complex as a transducer of signaling pathways, one would expect that the identification of specific signal transduction from gene expression data may allow isolating pathways that are correlated with cellular proliferation and prognosis. Indeed, recent advancement of bioinformatics has enabled to identify the lists of pathways activated or inactivated in cancer [9]. We recently combined the SAGE data with bioinformatics tools to identify the lipogenic pathway activated in a subset of HCC (Yamashita, T.; Honda, M.; Takatori, H.; Nishino, R. and Kaneko, S., manuscript in submission). Deactivation of lipogenic pathway resulted in the inhibition of cell proliferation and caused apoptosis *in vitro*. Furthermore, we identified that the overexpression of transcription factor regulating the lipogenic pathway (Sterol Regulatory Element Binding Protein 1; SREBP-1) correlated with poor prognosis, indicating the usefulness of this approach for detection of biomarker pathways. Similarly, a recent study has explored SAGE data to identify the subgroups of estrogen receptor-positive breast cancers with different metabolic activities that may be a determinant of prognosis [67]. Thus, pathway analysis of gene expression in cancer appears a promising method to decipher the mechanism of cell proliferation that is linked to poor prognosis.

Discovering Metastasis-Related Genes

As tumors progress to increase malignancy, they obtain the ability to invade into surrounding tissues to form new tumors at sites distinct from the primary tumors called metastasis. The process of cancer metastasis is considered as the sequential steps of the invasion-metastasis cascades, including local invasiveness, intravasation into and transport through the circulation, extravasation, formation of a micro-metastasis, and colonization [68, 69]. The molecular mechanisms involved in this process are complex and influenced by various host and tumor factors, including cell-cell and cell-matrix adhesion, degradation of extracellular matrix, hypoxia, and various growth factors and chemoattractants. Since metastasis accounts for about 90% of cancer death in

solid tumors [70], investigation of gene signatures of metastatic and non-metastatic tumors will provide molecular clues for diagnosis of cancer with poor prognosis. Microarray analysis has been extensively used to examine the global gene expression changes in metastatic and non-metastatic cancers [71]. For example, analyses of metastatic and non-metastatic breast cancers have provided the metastatic gene signature that could predict the survival of breast cancer patients [12, 72]. Similarly, analysis of metastatic and non-metastatic HCC patients has provided the gene signature that could distinguish metastatic HCC from non-metastatic even using primary tumor tissues [13]. These data suggested that the transcriptional alterations associated with metastasis are highly represented in their primary tumors, suggesting the utility of this approach for stratification of patients' prognoses. Furthermore, three groups recently utilized SAGE approach to uncover the signatures of breast cancer, thyroid cancer, and melanomas that are metastatic to the lymph nodes [73-75]. Although these studies have shown striking similarities of gene expression patterns between primary and metastatic tumors, they have successfully provided candidate genes associated with metastasis such as *HOXC10*, *LIMD2*, and *UBC9* in breast cancer, papillary thyroid cancer, and melanoma, respectively. Interestingly, all these studies suggested the activation of ATPase transporters or ATP-binding cassette (ABC) multidrug transporters in metastatic cancers, suggesting the involvement of these transporters on metastasis. Investigation of the functional role of these genes may provide profound insight into molecular portraits of metastatic cancer cells within primary tumors.

Predicting the Drug Response

Although cancer patients diagnosed at advanced stages may receive chemo/radiation therapies, it is hard to predict the responses in each cancer patient prior to treatment, mainly due to the lack of specific biomarkers. Estrogen receptors and HER-2 are good examples that have predictive information on outcome and sensitivity to a particular treatment in breast cancer. Identification of drug sensitivity markers using gene expression profiling may stratify patients who do (or do not) respond to the particular treatment with distinct prognosis. A cytogenetic study of anaplastic oligodendrogliomas indicated the strong association of allelic losses of chromosome 1p and 19q with longer overall survival and good chemo sensitivity, suggesting the utility of genetic analysis for therapeutic decisions [76]. Similarly, microarray studies have identified the 70-gene signature that could predict high risk groups of breast cancer recurrence who may have the survival benefits by adjuvant chemotherapy [77, 78]. Its prospective validation is currently ongoing through the MINDACT (Microarray in Node-Negative Disease May Avoid Chemotherapy) trial [79]. Gene expression profiling has also provided the mechanistic insights into cellular chemo sensitivity. A recent SAGE study has clarified the overexpression of extracellular matrix genes including *COL6A3* in cisplatin-resistant ovarian cancer cells [80]. Furthermore, an independent SAGE study to compare the gene expression profiles of various tumors with different chemo resistance has shown the differential expression of the extracellular matrix proteins [81]. Indeed, a role for tumor-matrix interactions in the acquisition of drug resistance has recently emerged [82]. SAGE studies have provided new

insights into the function of extracellular matrix proteins on tumor intractability to chemotherapy, and may provide the potential targets for elimination of tumors by inhibiting these targets in combination with chemotherapy.

As described above, enormous amounts of gene expression data generated by SAGE can be exploited to facilitate the identification of useful biomarkers/targets for treatment of cancer. The identified biomarkers may help subgrouping cancer patients with poor prognosis, although rigorous validation studies are clearly required using large-size patients' cohorts.

4. FUTURE GOALS

One of the fascinating advantages of using SAGE in cancer research is to identify the completely novel genes previously not detected that are altered in cancer. Certainly, we often experience the SAGE tags that can not match to the human genome even after the HGP and Celera genomics had provided the reliable human genome sequence data. Some of these tags have been mapped to the virus genome such as human papilloma virus and hepatitis B virus (Yamashita, T.; Honda, M.; Takatori, H.; Nishino, R. and Kaneko, S., manuscript in submission) [83], but the origin of many unknown tags is still uncertain. Recently, all publicly available human LongSAGE tags were investigated to map on human genomes [84]. Strikingly, the results have shown that about 36% of transcripts can not map to the human genome, and 58% of these can not be explained by polymorphisms, exon-intron boundaries, poly-A tail, and contamination of mouse transcripts (derived from SAGE libraries of human ES cell lines co-cultured with mouse embryonic fibroblasts). They have also identified that 31% of tags mapped to the genome are located outside of annotated exons, suggesting that these genes are still not annotated and could be candidates of novel genes. Similar results were obtained by the analysis of LongSAGE libraries of the human brain tissues [85]. Thus, a greater part of the transcripts unable to map to the human genome remains obscure, and about one-third of the transcripts mapped to the human genome is still not annotated. Future studies are clearly required to investigate whether these unmapped tags are derived from simple sequence errors, sequences correlated with unknown diversities of human genomes, or unknown peculiar transcriptional programs to generate unspecified transcripts.

Majority of gene expression profiling studies have been performed using bulk tumor tissues that are composed of various cell types such as epithelial, endothelial, mesenchymal, and inflammatory cells, as depicted in Fig. (2). Therefore, identified gene signatures may reflect the altered cellular heterogeneity rather than gene expression changes in tumor epithelial cells. Through SAGE analysis of purified various types of normal and tumor cells, Kornelia Polyak and her group have provided the strong evidence of tumor-microenvironment interaction in invasive breast cancer [86]. Although they found genetic alterations only in tumor epithelial cells, they identified dramatic gene expression changes in stromal cells that affect the metastatic ability of transformed tumor epithelial cells. Similarly, recent studies have successfully shown the differential gene expression between normal and tumor vascular endothelium that can be

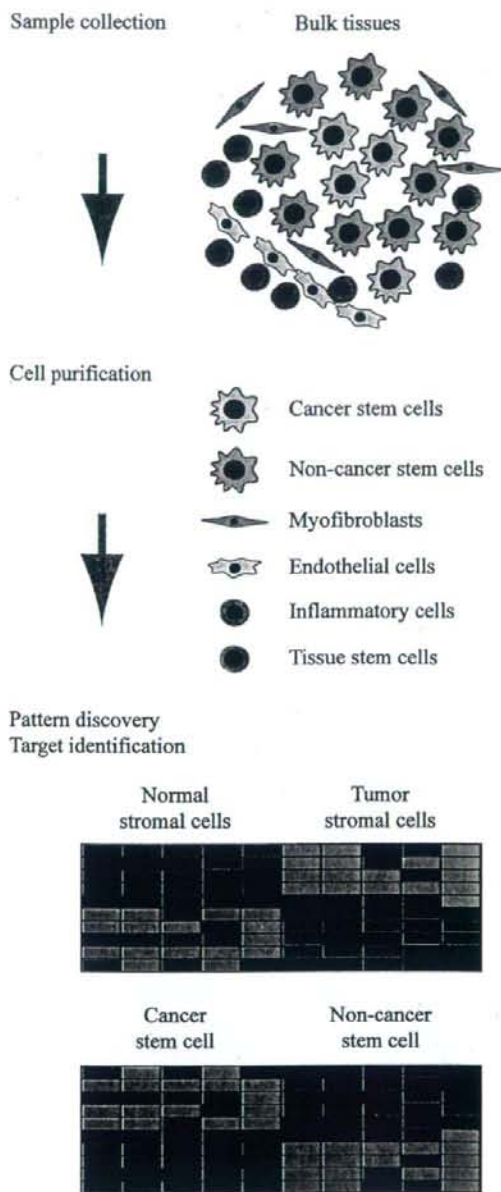


Fig. (2). Deciphering the cancer-microenvironment interaction in bulk tumor tissues by cell purification.

utilized for targeting tumor-specific vascular endothelial cells [87, 88]. These studies have accentuated the concept that cancers are heterogeneous cellular entities whose growth is dependent on reciprocal interactions between tumor cells and the dynamic microenvironment in which they reside [82]. Transcriptome studies of tumor stromal cells as well as

epithelial cells will lead to the discovery of underlying molecular targets in cancer-microenvironment interaction for eradication of tumors.

Cellular heterogeneity exists not only in stromal cells but also in purified tumor epithelial cells. Cancer stem cell concept, a subset of cells with stem cell features is indispensable for the development of tumor, has recently revived by the advancement of stem cell biology [89]. Accumulating evidence suggests the role of cancer stem cells on tumorigenesis, metastasis, and resistance to chemo/radiation, implicating the importance of targeting cancer stem cell population for elimination of tumor [90]. Although critics question the validity of the cancer stem cell hypothesis, comprehensive transcriptome studies of normal and putative cancer stem cells as well as non-cancer stem cells may clarify the key signaling pathways responsible for the stemness of these cells [91], and will provide clues for elimination of tumors in future.

ABBREVIATIONS

SAGE	=	Serial analysis of gene expression
HGP	=	Human genome project
RT-PCR	=	Reverse transcription-polymerase chain reaction
EST	=	Expressed sequence tag
HCC	=	Hepatocellular carcinoma
APC	=	Adenomatous polyposis coli
TGF- β	=	Transforming growth factor- β
VHL	=	Von Hippel-Lindau
PPAR	=	Peroxisome proliferator-activated receptor
BTB/POZ	=	Broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger
SREBP-1	=	Sterol regulatory element binding protein 1
ABC	=	ATP-binding cassette
MINDACT	=	Microarray in node-negative disease may avoid chemotherapy

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Comparative Analysis of Proteome and Transcriptome in Human Hepatocellular Carcinoma using 2D-DIGE and SAGE

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Abstract Proteome analysis of human hepatocellular carcinoma was conducted using two-dimensional difference gel electrophoresis, and the protein expression profiles were compared to the mRNA expression profiles made from serial analysis of gene expression (SAGE) in identical samples from a single patient. Image-to-image analysis of protein abundances together with protein identification by peptide mass fingerprinting yielded the protein expression profiles. A total of 188 proteins were identified, and the expression profiles of 164 proteins which had the corresponding SAGE data were compared to the mRNA expression profiles. Among them, 40 proteins showed significant differences in the mRNA expression levels between non HCC and HCC. We compared expression changes of proteins with those of mRNAs. We found that the expression tendency of 24 proteins were similar to that of mRNA, whereas 16 proteins showed different or opposite tendency to the mRNA expression.

Keywords Hepatocellular carcinoma · Proteome · Two-dimensional difference gel electrophoresis · Serial analysis of gene expression

Abbreviations

HCC	Hepatocellular carcinoma
SAGE	Serial analysis of gene expression
2-DE	Two-dimensional gel electrophoresis
2D-DIGE	Two-dimensional difference gel electrophoresis
IS	Internal standard
PMSF	Phenylmethyl sulfonyl fluoride
TFA	Trifluoroacetic acid
ACN	Acetonitrile
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
LC-ESI-IT	Liquid chromatography-electrospray ion trap tandem mass spectrometry
MS/MS	Mass spectrometry
PMF	Peptide mass fingerprinting

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1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and a leading cause of death in Africa and Asia [16]. Although several major risks related to HCC, such as hepatitis B and/or hepatitis C virus infection, aflatoxin B1 exposure, and alcohol drinking, and genetic defects, have been revealed [11], the molecular mechanisms leading to the initiation and progression of HCC are not well-known. To find the molecular bases of hepatocarcinogenesis, comprehensive gene expression analyses have been conducted using many systems such as hepatoma cell lines [8] and tissue samples [7]. Iizuka et al. [7] reported the differential gene expression in distinct

virologic types of HCC using oligonucleotide microarray, and they showed that 89 genes were expressed differentially between hepatitis B virus infected HCCs associated with liver cirrhosis and those not associated with liver cirrhosis.

The serial analysis of gene expression (SAGE) technique is thought to be an appropriate technique for evaluating thousands of gene expressions quantitatively, and has been used frequently to profile transcriptome [17]. We have previously done the comprehensive mRNA expression analysis in moderately differentiated HCC using SAGE. We found that many genes were expressed differentially between HCC and non HCC; INF-gamma inducible genes, superoxide dismutase 2, DEAD/H box polypeptide 5 and so on were up-regulated, whereas cytochrome P450 IIIA4 and IIA7, organic cation transporter 1 were down-regulated in HCC [22]. These genomic approaches have yielded global gene expression profiles in HCC and identified a number of candidate genes as possible biomarkers for cancer staging, prediction of prognosis, and treatment selection [13, 18]. Still, many researchers think that the proteomic study might reinforce our understanding of the molecular events accompanying HCC development because proteins rather than mRNA are the major effectors of cellular and tissue functions. It is generally accepted that mRNA and protein expression do not always correlate [4, 6], thus, protein expression analysis, which could complement the available mRNA data, is also important to understand the molecular mechanisms of HCC development.

Several proteomic researches have been performed so far on HCC using various protein separation technologies such as 2-DE followed by protein identification by mass spectrometry. The technique of two-dimensional difference gel electrophoresis (2D-DIGE), developed by Unlu et al. [21], is one of the major advances in quantitative proteomics. In 2D-DIGE, different samples pre-labeled with mass- and charge-matched fluorescent cyanine dyes, Cy3 and Cy5, are separated on the same 2D gel together with the internal standard prepared by mixing equal amounts of all samples and labeled with a third cyanine dye, Cy2. The labeled samples and internal standard are then mixed and fractionated on the same 2D gel. Coseparation of different samples on the same gel suppresses subtle changes in experimental conditions, thus enabling accurate spot detection and matching. The internal standard run on the background of all gels also facilitates gel-to-gel spot matching and allows the derivation of statistically reliable comparisons of protein abundances [1]. Recently several groups have utilized 2D-DIGE to examine protein expression changes in HCC samples [9, 10], whereas, there are few reports describing gene and protein expression profiles of the same HCC sample simultaneously [17].

We have previously analyzed gene expression changes between HCC and the adjacent non-tumor tissue extirpated from the same patient. As the patient was negative for hepatitis B and hepatitis C virus infection, we thought that the results should primarily reflect gene and protein expression changes involved in carcinogenesis. In order to complement our SAGE results, we performed proteomic research using 2D-DIGE and we compared the expression profile of proteins with that of mRNAs directly in the identical sample from a single patient.

2 Materials and Methods

2.1 HCC Sample

A 57-year-old male was admitted to the Kanazawa University Hospital and received hepatic lobectomy for treatment of a solitary HCC. The HCC sample and adjacent non-tumor liver sample were snap frozen in liquid nitrogen, and used for SAGE and 2-DE analysis. The HCC and non-tumor samples were histologically diagnosed as moderately differentiated HCC and mild hepatic fibrosis without active hepatitis, respectively. Serological tests of hepatitis B surface antigen (DAINABOT, Tokyo, Japan), hepatitis C virus antibodies (Roche Diagnostic Systems, Branchburg, NJ), and hepatitis C virus RNA by Amplicore analysis (Roche Diagnostic Systems) showed negative. Therefore, gene and protein expression change through viral infection, such as HBV [23] and HCV [3], was negligible, and the expression change together with carcinogenesis was shown clearly. All strategies used for gene expression and protein expression analysis were approved by the ethical committee of Kanazawa University Hospital.

2.2 Long SAGE

Long SAGE was performed as described previously [15]. Briefly, total RNA was purified from each homogenized tissue sample using a Totally RNA extraction kit (Ambion, Inc., Austin, TX), and polyadenylated RNA was isolated by a MicroPoly (A) Pure kit (Ambion). A total of 2.5 µg mRNA per sample was used for construction of Long SAGE libraries. Libraries were randomly sequenced at the Genomic Research Center (Shimadzu-Biotechnology, Kyoto, Japan), and the sequence files were analyzed with SAGE 2000 software. The size of each library was normalized to 300,000 transcripts per library, and the abundance of transcripts was compared by SAGE 2000 software. Monte Carlo analysis was performed to determine the statistical significance of obtaining a difference in expression in each transcript by SAGE 2000 software.

Each SAGE tag was annotated using a gene-mapping web site (<http://www.ncbi.nlm.nih.gov/SAGE/index.cgi>).

2.3 Protein Labeling with Cyanine Dye

Sample for 2-DE was homogenized with lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.8 μ M aprotinin, 15 μ M pepstatin, 0.1 mM PMSF, 0.5 mM EDTA, 30 mM Tris-HCl, pH 8.5) and then centrifuged at 13,000 rpm 20 min at 4 °C. The supernatants were removed and used as protein samples. The protein concentrations were determined with a protein assay reagent (Bio-Rad) according to the manufacture. The protein sample of tumor tissue was designated as HCC sample and the protein sample of noncancerous liver was designated as non HCC sample.

The experimental strategy is shown in Fig. 1. The non HCC sample (50 μ g) was labeled with Cy3 dye, and the HCC sample (50 μ g) was labeled with Cy5 dye. The labeled samples were combined and separated on 2-DE gels together with the internal standard (IS), which was prepared by mixing 25 μ g of non HCC and HCC samples, and labeled with Cy2. Labeling reactions were carried out according to the manufacture. Each sample was labeled with 400 pmol of CyDye (GE Healthcare) on ice for 30 min in the dark and reactions were stopped by adding 1 μ l of 10 mM lysine. The CyDye-labeled samples (non HCC, HCC, and IS) were mixed and left for 10 min on ice in the dark. The mixtures were added to an equal volume of the sample buffer (7 M urea, 2 M thiourea, 1% v/v IPG buffer (GE Healthcare), 2.4% v/v Destreak Reagent (GE Healthcare), 4% w/v CHAPS). Mixed samples were then adjusted to 450 μ L with the rehydration buffer (7 M urea, 2 M thiourea, 0.5% v/v IPG buffer (GE Healthcare), 1.2%

v/v Destreak Reagent (GE Healthcare), 4% w/v CHAPS, trace of bromophenol blue).

2.4 Analytical and Preparative 2-DE for 2D DIGE Analyses

Analytical 2-DE was performed as follows: The labeled protein samples were electrophoresed in the first dimension on IPG gels (Immobiline DryStrip, GE Healthcare, pH 3–10 or pH 4–7, 24 cm) using the IPGphor system (GE Healthcare). After rehydration at 20 °C for 12 h, IEF was carried out at 500 V for 500 Vh, at 1,000 V for 1,000 Vh, and at 8,000 V for 70,000 Vh in the dark. The gel strips were equilibrated in 12 mL of the equilibration buffer A (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT, trace of bromophenol blue) for 15 min with gently shaking, and then were equilibrated in 12 mL of the equilibration buffer B (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% w/v iodoacetamide, trace of bromophenol blue) for 15 min with gently shaking. The equilibrated strips were loaded on the top of 12.5% SDS-polyacrylamide gels (24 \times 20 cm) and sealed with 0.5% w/v agarose. 2D separation was performed overnight at 1 W/gel using Ettan DALTII (GE Healthcare). After 2-DE, gels were scanned with a Typhoon 9410 scanner (GE Healthcare) using filters conformable to each dye's excitation and emission wavelength, and then to analyze the high molecular region with high resolution, the scanned gels were run again at 5 W/gel for a further 16 h (extended gel). After this additional electrophoresis, the extended gels were scanned with the Typhoon scanner as described above, and then the twice-scanned gels were stained with Silver Staining Reagent (GE Healthcare) without glutaraldehyde. Samples were run in triplicate to obtain statistically reasonable results.

To obtain an adequate amount of the proteins from the individual spots for protein identification, 400 μ g of the proteins extracted from the non HCC or HCC were separately run on 2-DE gels as described above. After 2-DE, these preparative gels were fixed in 10% v/v methanol and 7% v/v acetic acid aqueous solution for 30 min and stained with a fluorescent dye, SYPRO Ruby (Invitrogen) overnight at room temperature according to the manufacturer. The gels were washed twice with 10% v/v methanol and 7% v/v acetic acid for 30 min and then washed three times with distilled water for 5 min. The washed gels were scanned with the Typhoon scanner.

To detect phosphoproteins, 400 μ g samples from non HCC and HCC were separately run on 2-DE gels. The gels were fixed on 50% methanol and 10% acetic acid overnight at room temperature. The gels were then washed three times, for 15 min each time with distilled water and stained

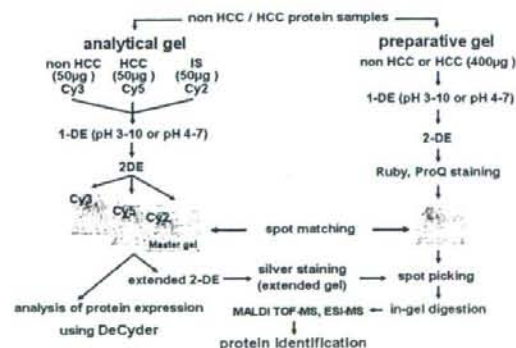


Fig. 1 Outline of 2D-DIGE analysis. After being labeled with the respective CyDye, samples were combined with the Cy2-labeled internal standard (IS) and separated on 2-DE. The experiment was triplicate to obtain numerical; data sufficient for statistical analysis

with ProQ Diamond Phosphoprotein Gel Stain (Invitrogen) for 1 h in the dark. The gels were then washed with a destaining solution (Invitrogen) three times for 30 min each time at room temperature. After obtaining the images, the ProQ Diamond-staining gels were counterstained with SYPRO Ruby to visualize the total proteins as described above.

2.5 Analysis of Gel Images

For CyDye-labeled analytical gels, a 488 nm laser and 520 nm emission filters were used for Cy2 images, a 532 nm laser and 580 nm emission filters for Cy3 images, and a 633 nm laser and 670 nm emission filters for Cy5 images. Preparative gels stained with SYPRO Ruby were scanned with a 457 nm laser and 610 nm emission filters. All gel images were obtained at 100 μ m resolution and processed using ImageQuant (GE Healthcare) prior to image analysis. The analysis of gel images was performed with the DeCyder software (GE Healthcare). The Cy2, Cy3, and Cy5 images of the same gel were electrically merged and the proteins were detected as Cy3/Cy2 and Cy5/Cy2 spot pairs. For each spot pair, the normalized ratio of spot volume relative to IS (Cy3: Cy2 or Cy5: Cy2) was calculated and analyzed statistically. The statistical differences in each normalized spot volume between non HCC and HCC were analyzed by the Student's paired *t*-test using DeCyder BVA (GE Healthcare), and *p* value ≤ 0.05 was considered as significant change. For protein identification by PMF, the spots visualized with SYPRO Ruby were matched to those on CyDye images.

2.6 Protein Identification

The protein spots were excised from the SYPRO Ruby stained gels using the Ettan spot picker (GE Healthcare) or manually excised from the silver stained gels. The excised protein spots collected in 96-well plates were digested in gel with porcine trypsin (Promega) using an automatic digestion robot, ProGest (Genomic Solutions), according to the manufacture. For MALDI-TOF analysis using Voyager-DE STR (Applied Biosystems), the digested and dried peptides were dissolved in 5 μ L of 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile (ACN). Aliquots (1 μ L) were spotted on MALDI target and then 1 μ L of α -cyano-4-hydroxycinnamic acid (5 mg/mL in 0.1% TFA in 60% ACN) was immediately added. MALDI-TOF analysis was carried out in reflector mode in the mass range of 850–3,000 Da. A database search was carried out against *Homo sapiens* category of NCBI entries using MS-Fit (Protein Prospector) or ProFound (http://www.129.85.19.192/profound_bin/WebProFound.exe). The search parameters allowed for carbamidomethylation of cysteine, partial oxidation of methionine, ± 0.3 Da for

mass tolerance, and one missed cleavage per peptide. For LC-ESI-IT MS/MS analysis using LCQ Deca XP (Thermo Electron), the digested and dried peptide samples were dissolved in 10 μ L of 0.1% TFA in 2% ACN. The samples were loaded on a C18 silica gel capillary column (Magic C18, 50 \times 0.2 mm), and the elution from the column was directly connected through a sprayer to an ESI-IT MS. Mobile phase A was 2% ACN containing 0.1% formic acid, and mobile phase B was 90% ACN containing 0.1% formic acid. A linear gradient from 5% to 65% of concentration B was applied to elute peptides. The ESI-IT MS was operated in positive ion mode over the range of 350–2000 (*m/z*) and the database search was carried out against *Homo sapiens* category of NCBI entries using MASCOT (Matrixscience). The identified proteins were classified according to their molecular functions and the cellular components into categories described by Gene Ontology Consortium (<http://www.geneontology.org/index.shtml>) using BioCompass software (NEC Corp.).

3 Results

3.1 2-DE and Protein Identification

Labeled protein samples were separated on 2-DE, and the fluorescence images were obtained (Fig. 2). In parallel to the analytical gels, samples prepared from non HCC and HCC tissues were separately run on the preparative gels for protein identification. The proteins were visualized with SYPRO Ruby, and images were directly matched to CyDye images of analytical gels. Approximately 1,100 spots were analyzed, and 361 spots representing 183 proteins were determined (Fig. 3). Three proteins were excluded from the following analysis because of ambiguous spot matching between analytical gels and preparative gels. The extended gel images mentioned in Sect. 2.4 were analyzed with DeCyder software, and about 20% of analytical spots, whose expression levels between HCC and non HCC were altered by more than 1.8-fold (230 protein spots), were selected for PMF analysis. After silver staining, these 230 spots were manually excised and 143 spots representing 66 proteins were identified. Eight proteins were newly detected in extended gels. As a result, total of 188 proteins were identified from Ruby and silver stained gels (Supplementary Table 1). The identified proteins and mRNAs were classified according to their molecular functions and the cellular components into categories described by Gene Ontology Consortium (Fig. 4a, b). As for molecular functions, about 60% of the identified proteins were classified as catalytic activity, such as metabolic enzymes, and 20% of them were classified as binding activity, such as heat shock proteins (Fig. 4a). The major cellular components of

Fig. 2 Merged view of 2D-DIGE images of non HCC and HCC tissues samples. Protein samples (50 μ g) extracted from non HCC labeled with Cy3 and HCC labeled with Cy5 were electrofocused on a 24 cm pH 3–10 or pH 4–7 IPG strips and then separated by SDS-PAGE on a 12.5% gel over night. The more electrophoresis was performed to extend high molecular region. Red spots and green spots represent abundant and less abundant proteins in HCC, respectively. (a) pH 3–10, (b) pH 4–7, (c) pH 3–10 extend, (d) pH 4–7 extend

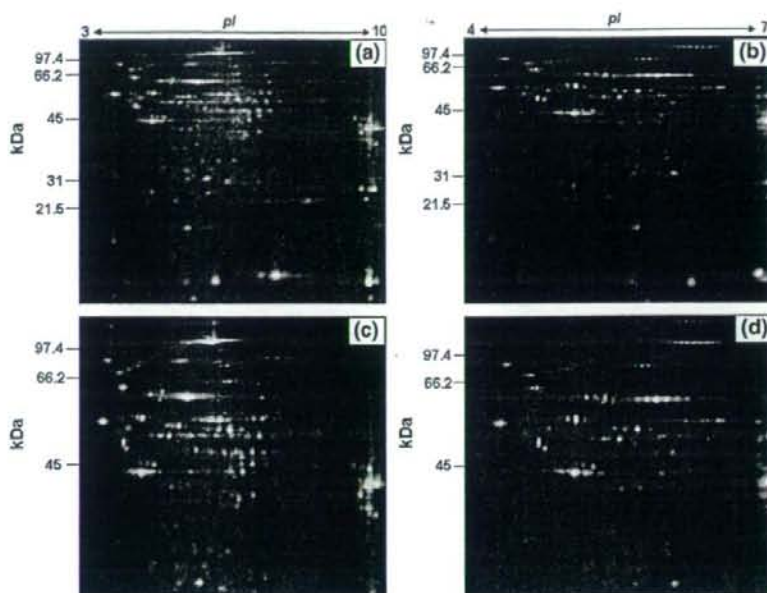
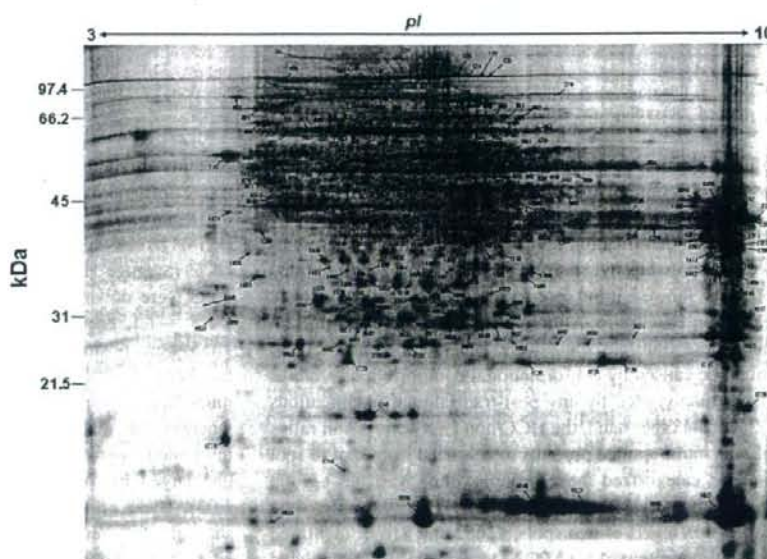


Fig. 3 2-DE map of non HCC proteins (pH 3–10). Proteins were determined through PMF using preparative gels run in parallel with analytical gels. Identified spots were projected onto the analytical image of non HCC sample. Annotations in the gel refer to the Spot ID in Supplementary Table 1



the identified proteins were mitochondrion (40%), cytosol (20%), nucleus (10%), and endoplasmic reticulum (10%; Fig. 4b). It was noticed that ribosome and plasma membrane were major cellular components of identified mRNAs (Fig. 4d), whereas identified proteins localized at ribosome and plasma membrane were rare (Fig. 4b).

3.2 Protein Expression Analysis

Using fluorescence images of proteins from non HCC and HCC samples, the normalized spot volume of protein abundance for each spot was calculated relative to the internal standard using DeCyder software. We performed

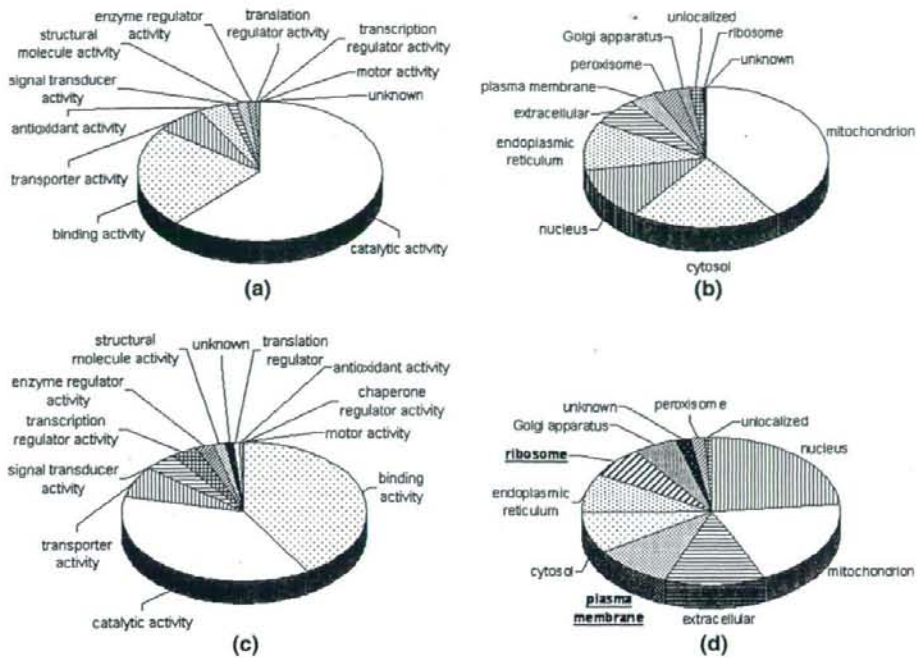


Fig. 4 Functional categorization and cellular components of identified proteins from 2-DE and identified genes from SAGE. The molecular functions and cellular components classification were performed according to criteria described by Gene Ontology

Consortium using BioCompass. (a) molecular functions of identified proteins, (b) cellular components of identified proteins, (c) molecular functions of identified genes, (d) cellular components of identified genes

Student's paired *t*-test ($p \leq 0.05$) to filter the protein spots which differentially expressed between non HCC and HCC, using 2-DE gel images run in triplicate.

Protein identification revealed that a number of proteins were expressed by multiple spots. Most multi-spotted proteins showed little spot-to-spot variations in averaged HCC/non HCC ratio. Although we do not know at present how these multiple spots were generated, some of them might be caused by conformational equilibrium of proteins rather than caused by any post-translational modifications [2]. On the other hand, the HCC/non HCC expression ratios of nine multi-spotted proteins were varied from spot to spot and we categorized these proteins into "variable" group (Supplementary Table 1, Fig. 5). For example, the expression levels of two spots, #1682 and #1693, identified as apolipoprotein A1 (APOA1) seemed to be regulated oppositely between HCC and non HCC. While the expression of spot #1682 was decreased in HCC, the expression of spot #1693 was increased in HCC (Fig. 5a, b). The spots #302 and #377, both identified as heat shock protein 90 kDa beta (GRP94), showed different molecular mass and pI. The expression levels of these spots seemed to be differently regulated (Fig. 5c, d). These variable group

proteins might be caused by multiple isoforms or post-transcriptional modifications. For example, the spots #644, #654, and #655 were identified as phosphoglucomutase 1 (PGM1). The spot of #644 was slightly up-regulated in HCC (average HCC/non HCC ratio: 1.55), and #654 and #655 were down-regulated and unchanged (average ratio: -1.8, -1.1, each) in HCC, respectively. Both #654 and #655 were positively stained with ProQ Diamond which has been shown to detect phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins [12, 19], whereas the #644 was negative for ProQ (Fig. 6). The integrated spot volume of #644, #654, and #655 were unchanged between non HCC and HCC (average ratio: 1.03). These observations suggest that the PGM1 does not show the different expression level between non HCC and HCC, but exhibits altered phosphorylation level in HCC.

3.3 Comparison of Protein and mRNA Expression Profile

The expression pattern of 188 proteins was compared with that of mRNA expression pattern obtained from the same tissue samples, and a total of 164 proteins had the

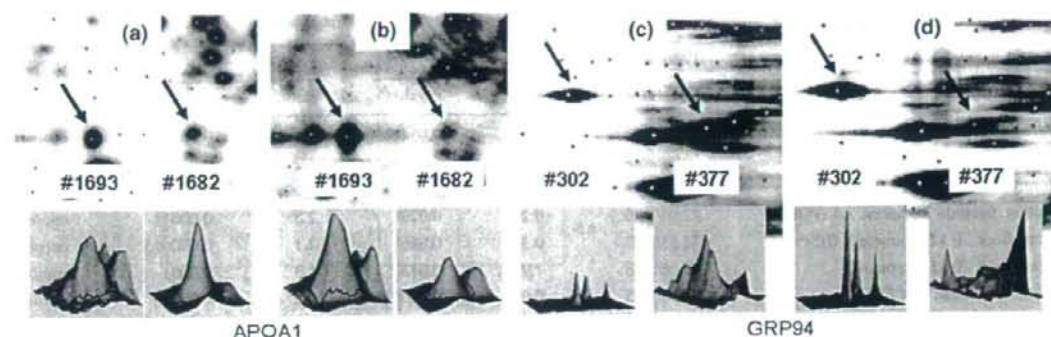
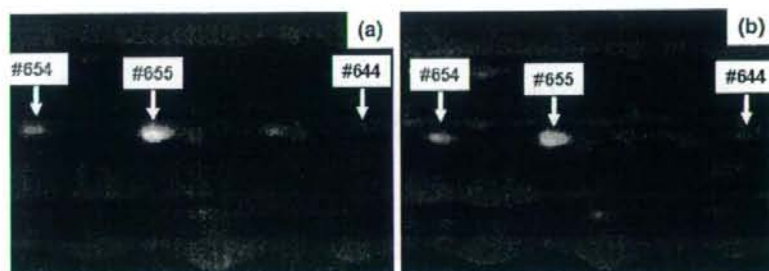


Fig. 5 Protein expressed as multiple spots. Apolipoprotein A1 (APOA1) in non HCC (a), in HCC (b), and heat shock protein 90 kDa beta (GRP94) in non HCC (c), in HCC (d) were detected as

multi-spotted protein. Magnified images of selected spots and 3-D views of selected spots were shown

Fig. 6 Detection of phosphoglucomutase 1 with Ruby and ProQ Diamond. Phosphoglucomutase 1 (PGM1) was detected as three spots (#644, #654, and #655). The spot #644 was stained with Ruby (yellow) only, and the spot 654 and 655 were stained with Ruby and ProQ Diamonds (blue green) in non HCC (a) and HCC (b) gels



corresponding mRNA expression data. The protein expression abundance between non HCC and HCC was calculated using the average spot volume, and the statistical differences were analyzed by the Student's paired *t*-test using DeCyder BVA. The spot volume of a multi-spotted protein was indicated as a total volume by integrating the intensities of multiple spots. The mRNA expression abundance was calculated the ratio of the SAGE tag counts, and the SAGE tag counts were evaluated by Monte Carlo analysis whether each tag ratio between HCC and non HCC was statistically significant or not. Consequently, the mRNA levels for forty proteins out of 164 were shown to be altered significantly ($p \leq 0.05$) between non HCC and HCC (Table 1). These proteins were used to clarify the difference of transcriptional and translational regulation in HCC. The HCC/non HCC expression ratio for each protein was plotted against the mRNA ratio in log scale in Fig. 7a, where a positive value indicates increased expression in HCC and a negative ratio indicates reduced expression. We defined the protein and mRNA showed same (increase or reduce) expression change as similar group, and showed opposite expression as opposite group, respectively. The protein expression did not show significant change ($p > 0.05$) was defined as different group. Above that

classification, 24 proteins showed similar protein and mRNA expression tendency between non HCC and HCC (Fig. 7a, open circles).

On the other hand, 11 proteins showed the opposite (Fig. 7a, closed circles) and 5 proteins showed the different (Fig. 7a, half-closed circles) expression patterns, respectively. Thus, about 60% of 40 proteins classified as similar group, 30% as opposite group, and 10% as different group (Fig. 7b). The similar and the opposite group proteins were classified according to their molecular functions and the cellular components (Fig. 8). The ratio of members categorized into each function differs slightly between the similar (Fig. 8a) and the opposite (Fig. 8c) group proteins. On the other hand, the result of classification by cellular components were apparently different each other (Fig. 8b, d).

4 Discussion

In the present study, a paired sample of HCC and non HCC from the single patient at the proteome and transcriptome level were compared. We used 188 identified proteins expression data and 4235 unique mRNA expression data,

Table 1 Proteins of significant mRNA expression abundance

Protein name	Theoretical MW (kDa)/pI	mRNA		Protein		Group ^e
		Ratio ^a	<i>p</i> value ^b	Ratio ^c	<i>p</i> value ^d	
Carbamoyl-phosphate synthase 1	164.94/6.29	0.25	0.054	1.4	0.00046	opposite
Actinin, alpha4 (ACTN4)	105.29/5.3	2.1	0.014	1.2	0.013	similar
Formyltetrahydrofolate dehydrogenase	98.83/5.63	9	0.011	0.99	>0.05	different
Protein disulfide isomerase A4 (PDIA4)	72.93/4.96	0.2	0.029	2.5	0.00011	opposite
Heat shock 70 kDa protein 5 (HSPA5)	72.33/5.07	0.3	0.046	2.1	0.000013	opposite
Heat shock 70 kDa protein 8	71.08/5.28	1.9	0.013	1.3	0.0001	similar
Carboxylesterase 1 (CES1)	62.38/6.15	0.048	0.000013	0.55	0.00005	similar
Aldehyde dehydrogenase 4A1	61.72/8.25	3.5	0.016	1.01	>0.05	different
Glutamate dehydrogenase 1 (GLUD1)	61.4/7.66	0.1	0.000063	0.21	0.0000054	similar
Proline 4-hydroxylase (P4HB)	57.11/4.76	1.8	0.033	2.1	0.0000059	similar
ATP synthase H+ transporting	56.56/5.26	3	0.010	1.00	>0.05	different
Aldehyde dehydrogenase 9A1	54.7/5.7	0.2	0.029	0.84	0.00078	similar
Cytokeratin 8 (KRT8)	53.67/5.3	0.11	0.00011	0.41	0.00017	similar
Endothelial cell growth factor 1	49.96/5.36	3.5	0.016	0.43	0.00064	opposite
Heterogeneous nuclear ribonucleoprotein	46.04/5.38	0.17	0.017	0.98	>0.05	different
Betaine-homocysteine methyltransferase	45.43/6.41	10	0.0066	0.88	0.0091	opposite
Acyl-CoA dehydrogenase, C-2 to C-3	44.3/7.96	0.28	0.0012	0.51	0.00031	similar
Alanine-glyoxylate aminotransferase	43.39/8.61	0.33	0	0.78	0.044	similar
Acetyl-CoA acyltransferase 2	42.04/8.52	0.18	0	0.52	0.0007	similar
Actin beta (ACTB)	41.35/5.56	0.067	0.00029	0.74	0.000013	similar
Alcohol dehydrogenase 1B	39.84/8.53	0.18	0	0.44	0.000012	similar
Glyoxylate reductase	37.31/5.95	3.5	0.016	0.63	0.041	opposite
Aldo-keto reductase 1C3	36.85/8.06	3.3	0.047	1.5	0.025	similar
Malate dehydrogenase 1	36.62/5.92	0.33	0.023	1.2	0.00047	opposite
Apolipoprotein E (APOE)	36.25/5.6	7	0.037	1.9	0.013	similar
Lectin, galactoside-binding 4	36.03/9.21	10	0.0066	2.5	0.00045	similar
Malate dehydrogenase 2	35.5/8.92	0.3	0.046	0.73	0.00047	similar
Sulfotransferase family, cytosolic, 2A	33.95/5.71	0.25	0.0019	0.85	0.015	similar
Thiosulfate sulfurtransferase	33.43/6.77	0.38	0.027	0.97	>0.05	different
Peroxioredoxin 4 (PRDX4)	30.77/5.86	0.13	0.0039	1.2	0.032	opposite
Carbonyl reductase 1	30.37/8.55	4.5	0.032	1.5	0.000027	similar
Phosphoglycerate mutase 1	28.92/6.67	0.22	0.032	0.52	0.00016	similar
Proteasome activator subunit 1	28.72/5.78	0.14	0.037	1.4	0.0017	opposite
Glutathione-S-transferase omega 1	27.83/6.2	0.22	0.032	0.64	0.0000004	similar
Quinoid dihydropteridine reductase	26.02/6.9	0.2	0.029	0.78	0.00049	similar
Proteasome subunit alpha 2	25.99/6.9	0.17	0.000073	1.4	0.00035	opposite
Hemoglobin beta	16/6.74	0.23	0.00026	0.40	0.000034	similar
Hemoglobin alpha	15.17/8.7	0.4	0.019	0.44	0.000092	similar
Fatty acid binding protein 1	14.26/6.6	0.39	0	0.83	0.00027	similar
Cytochrome b5A (CYB5A)	11.27/5.02	0.15	0.0039	1.3	0.000062	opposite

^a mRNA ratio represents SAGE tag counts ratio of HCC/non HCC

^b SAGE *p* values were calculated by Monte Carlo analysis using SAGE 2000 software

^c Protein ratio represents the average spot volume ratio of HCC/non HCC

^d Protein *p* values were analyzed by the Student's paired *t*-test using DeCyder BVA software

^e Protein and mRNA expression change showed same tendency represents as similar. Protein and mRNA expression change showed opposite tendency represents as opposite. Protein expression showed no significant change represents as different

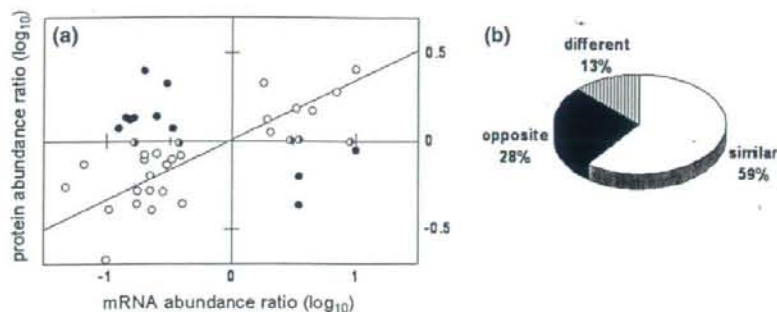


Fig. 7 Comparative analysis of protein and mRNA expression. (a) The log₁₀ values of the HCC/non HCC 2D-DIGE gel spot volume ratio were plotted against that of the HCC/non HCC SAGE tag counts ratio which showed statistical significance of obtaining a difference in expression. The open circles (○) represent similar expression tendency of protein and mRNA between non HCC and HCC. The

closed circles (●) showed opposite tendency, and the half-closed circles (◐) showed different tendency which represent SAGE ratio showed significant change but 2D-DIGE ratio did not between non HCC and HCC. (b) The pie chart represents the percentage of similar, opposite, and different tendency of 40 proteins

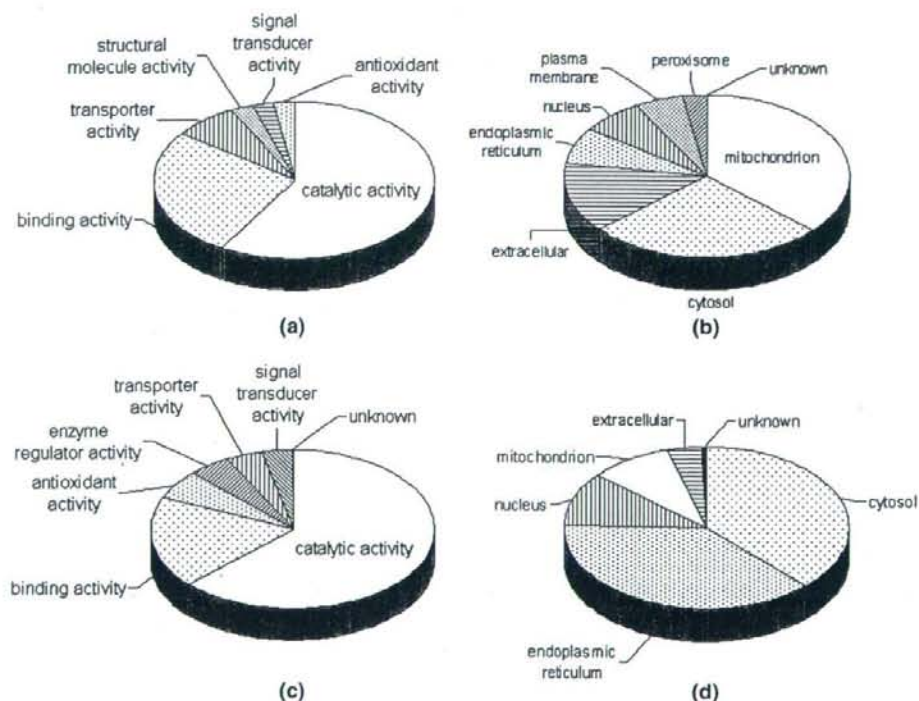


Fig. 8 Molecular functions and cellular components classification of the similar and the opposite proteins. The molecular functions and cellular components classification were performed according to criteria described by Gene Ontology Consortium using BioCompass.

(a) molecular functions of the similar proteins, (b) cellular components of the similar proteins, (c) molecular functions of the opposite proteins, (d) cellular component of the opposite proteins

eliminating multiple gene match tag, and protein data were linked to the corresponding mRNA data using GenBank accession, UniGene accession, and protein name. The

mRNA data for 24 proteins were not found in the SAGE data set (Supplementary Table 1). These mRNA data might be excluded as ambiguous identification of SAGE tag