

Fig. 8. *In situ* zymography of a mouse liver after (A) 5-week treatment with CCl₄ alone and (B, C) 1 week after BMC transplantation (BMT) with CCl₄ treatment for 5 weeks. (Original magnification, [A] ×40; [B] ×40, [C] ×100.)

tissue.^{22,23} Therefore, the increased expression of MMP-9 in this study was somehow related to the migration of BMCs to the inflammatory liver.

Film *in situ* zymography clearly showed that these MMP-9-positive cells possessed high gelatinolytic activity compared with the liver treated with CCl₄ alone. Thus, the BMCs that migrated acted in the degradation of liver fibrosis (fibrolysis).

According to our present data, increased expression of MMP-14 (MT1-MMP [membrane-type 1 matrix metalloproteinase]) will contribute to degrading interstitial collagens²⁴ to gelatin that MMP-9 can degrade, resulting in the regression of fibrosis (fibrolysis).

Recently, Koller et al.²⁵ reported that the expression of MMP-9 was increased with the migration of human CD34⁺ progenitor cells in CCl₄-treated NOD/SCID mice and that an inhibitor of MMP-9 reduced this migration. Thus, proteolytic activity seems to be necessary for the cell migration in addition to matrix degradation activity.

It seems to be very important how many cells can migrate into the damaged liver to degrade fibers, but a recent paper²⁶ reported little evidence of bone marrow-derived hepatocytes in the CCl₄-treated liver. However, the dose of CCl₄ was only 4% (0.08 mL/kg) of our dose (0.5 mL/kg), and the number of mice used was too small (1 or 2). The reason they did not see the BMCs that migrated is most likely due to the cessation of CCl₄ injection after BMC transplantation. Even in our experimental model,¹¹ the cessation of CCl₄ after BMC transplantation dramatically reduced the number of BMCs migrating into the

damaged liver (I.S., unpublished data, 2003). Thus, the extent of continuing liver damage may limit BMC migration to the liver with matrix degradation activity.

Transplanted BMCs differentiated into albumin-producing hepatocytes with an increased serum albumin level, and the degradation of the extracellular matrix may presumably lead to improved liver function resulting in better survival of mice with BMC transplantation compared to that of treated with CCl₄ alone, although only 1 transplantation of BMCs was performed.

As shown by double fluorescence, our data may also indicate that transplanted BMCs seem to become stellate cells, in agreement with a recent report,²⁷ although the number was very small in our experimental model. This result seems to be contradictory to our result of resolution of liver fibrosis by BMC transplantation because transdifferentiated stellate cells may produce collagens. Our preliminary results indicated reduced messenger RNA expression of type I procollagen, transforming growth factor- β 1 (TGF- β 1) and no change of hepatocyte growth factor messenger RNA expression in the liver 1 week after BMC transplantation compared with the liver treated with CCl₄ alone (I. Sakaida, unpublished data, 2003). As shown in Fig. 7, migrated BMCs seemed to reduce the fine network pattern of activated stellate cells. Thus, transplanted BMCs may affect activated stellate cells by reducing their number—*e.g.*, by leading them to apoptosis. However, further examinations are necessary to determine the exact relationship between BMCs and resident stellate cells.

Our recent data¹² indicated that the subpopulation of BMCs, nonhematopoietic cells in bone marrow, separated using an anti-Liv8 antibody, will transdifferentiate into hepatocytes in the liver damaged by CCl₄ induction. The present study clearly indicates that this subpopulation of BMCs is also responsible for the resolution of liver fibrosis (fibrolysis) induced by CCl₄ treatment.

In conclusion, the present study introduces a new concept for the treatment of liver fibrosis.

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Molecular signature associated with plasticity of bone marrow cell under persistent liver damage by self-organizing-map-based gene expression[☆]

Kaoru Omori^a, Shuji Terai^{a,*}, Tsuyoshi Ishikawa^a, Kouji Aoyama^a, Isao Sakaida^a, Hiroshi Nishina^b, Koh Shinoda^c, Shunji Uchimura^d, Yoshihiko Hamamoto^d, Kiwamu Okita^a

^aDepartment of Molecular Science and Applied Medicine (Gastroenterology and Hepatology), Yamaguchi University School of Medicine, Minami Kogushi 1-1-1, Ube, Yamaguchi 755 8505, Japan

^bDepartment of Physiological Chemistry, Graduate School of Pharmaceutical Science, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113 0033, Japan

^cDepartment of Neuroanatomy and Neuroscience, Yamaguchi University School of Medicine, Minami Kogushi 1-1-1, Ube, Yamaguchi 755 8505, Japan

^dDepartment of Computer Science and Systems Engineering, Faculty of Engineering, Yamaguchi University, Tokiwadai 2-16-1, Ube, Yamaguchi 755 8505, Japan

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Abstract The mechanism that regulates the plasticity of bone marrow cells (BMCs) into hepatocytes is poorly understood. We developed a green fluorescent protein/carbon tetrachloride model to find that BMC transplantation recovered liver damage. Serum albumin level and liver fibrosis were recovered by BMC transplantation. To understand the mechanism, we used DNA-chip technology to profile the change of transient gene expression before and after BMC transplantation. On the basis of gene expression with self-organizing map using specific equation, genes were classified into 153 clusters. The information is useful to understand the dramatic gene activation during the process of the plasticity of BMC.

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Keywords: Bone marrow cell; Plasticity; Regenerative Medicine; Gene expression; Microarray analysis; Self-organizing map; Liver regeneration

1. Introduction

Recently, several groups have reported the possible plasticity of bone marrow cells (BMC) to differentiate into a variety of non-hematopoietic cell lineages [1,2]. Ever since, the differentiation of BMC into hepatocytes in human was documented following a bone marrow transplantation from a man to a woman [3]. The mechanism of the plasticity of BMC was discussed whether that was occurred with cell fusion, nuclear reprogramming [4–6] or trans-differentiation [7,8]. We think both cell fusion and trans-differentiation might be important to understand the mechanism of BMC plasticity. On the other hand, in cardiovascular medicine, clinical research has been conducted to evaluate the use of BMCs in regenerating the myocardium and vessels, and some positive results have been obtained [9,10]. These findings suggest the usefulness of BMCs as the source of cells in developing the next-generation of treatment for liver regeneration [11]. We first tried to understand how we could use BMC to repair damaged liver. We have developed a model [named as a green fluorescent protein/carbon tetrachloride (GFP/CCl₄)] to evaluate the usefulness of BMC transplantation for damaged liver [12,13]. In this model, 0.5 ml/kg of carbon tetrachloride (CCl₄) is administered twice weekly to induce liver cirrhosis and then GFP-positive BMCs are transplanted through the causal vein [14]. Under continuous liver injury, immunostaining using anti-GFP antibodies [15] showed that GFP-positive BMCs migrated into the marginal area of the hepatic lobule starting from day 1 after BMC transplantation, and with time, while forming a hepatic cord towards the central vein, the distribution of GFP-positive BMCs expands [12,16]. Also, using Liv2, a hepatoblast-specific antibody that we developed [17], it has been shown that BMCs first trans-differentiate into Liv2-positive hepatoblasts and then differentiated into albumin-positive hepatocytes. Furthermore, the level of serum albumin significantly increases with time in recipient mice. Liver fibrosis induced by CCl₄ injection was recovered by BMC transplantation [18]. These findings suggest that this GFP/CCl₄ model can be used

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^{*} Corresponding author. Fax: +81-836-222-240.
E-mail address: terais@yamaguchi-u.ac.jp (S. Terai).

Abbreviations: BMC, bone marrow cell; SOM, self-organizing map; CCl₄, carbon tetrachloride; EGFP, enhanced GFP; GFP, green fluorescent protein; RT, reverse transcriptase; HNF4- α , hepatocyte nuclear factor 4 alpha; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; FAH, fumarylacetoacetate hydrolase; TNFR, tumor necrosis factor receptor; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; NumbL, Numblike; HOX, homeobox; GPI, glucose-6-phosphatase isomerase

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to understand the process of plasticity of BMCs under persistent liver damage condition. It is important to understand what had happened in GFP/CCl₄ model after BMC transplantation in mRNA level. DNA chips are recently developed tools used in genetic analyses [19]. While it is possible to obtain genetic data using DNA chips, the vast amount of information collected makes it difficult to precisely interpret the factors involved in the gene expression. Therefore, in the present study, patterns of global gene expression at different

times were compared between mice with BMC transplantation and those without. Self-organizing map (SOM) is a statistical technique that has been recently used in analyzing microarray data and, via this method, it is possible to visualize a vast amount of complicated and multidimensional data [20]. In this analysis, we made a specific equation to extract genes with expressions that altered in relation to BMC transplantation. Here, we present the results obtained from this study.

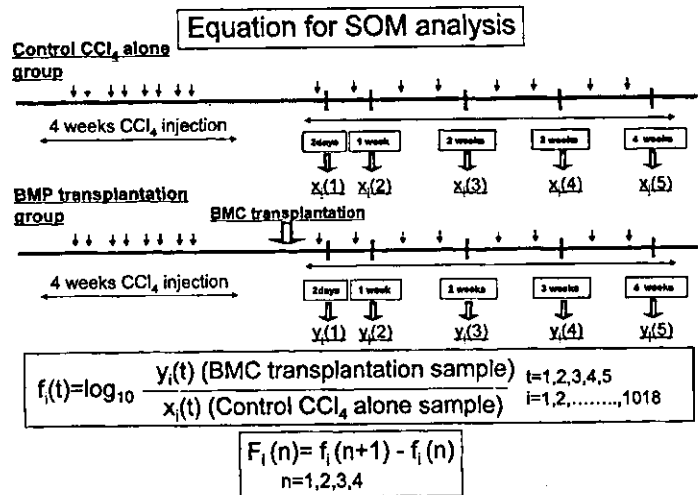


Fig. 1. Defined equation in this analysis. (1) Arrow indicates CCl₄ injection twice a week in GFP/CCl₄ model. We analyzed gene expression in each time point (2 days, 1 week, 2 weeks, 3 weeks, and 4 weeks). $y_i(t)$ showed the gene expression level of the liver after BMC transplantation. $x_i(t)$ showed the gene expression level of the liver CCl₄ alone injection group. We define $f_i(t): f_i(t) = \log_{10} y_i(t)/x_i(t)$. By using this, we succeeded in extracting the change of gene expression by BMC transplantation. We are interested in following the change of the values of $F_i(t) = f_i(t+1) - f_i(t)$ with the increase in t .

Table 1
Lists of primers for selected 13 genes

| Cluster | Gene name (Accession No.) | Primer – forward (5'–3') Primer – reverse (3'–5') | Target | Tm |
|---------|---------------------------|--|--------|----|
| 86 | c-kit (NM02099) | TCCAACGATGTGGGCAAGAG AATGAGCAGCGCGTGAA | 90 | 55 |
| 86 | FGP6 (M92415) | CATGGTCTATACCGGCCACA GGCTGCTGACATGAAACCAAAG | 88 | 63 |
| 125 | MMP2 (NM008610) | CCGTGATGTCAGCAAGTAGATG ATCCAGGAGTCTGCCGATGAG | 148 | 62 |
| 125 | MMP9 (NM013599) | ACGACATAGACGGCATCCAGTA TCGGCTGTGGTTCAGTTGTG | 90 | 53 |
| 92 | TIMP2 (NM011594) | ACACGCTTAGCATCACCCAGA TGTGACCCAGTCCATCCAGAG | 137 | 63 |
| 13 | HGF (NM010427) | CCCAAACATCCGAGTTGGCTAC TTCCCATGCCCACGATAACA | 84 | 63 |
| 1 | NumbL (NM010950) | TATGCAGCCTCCGTTTGTG GCGTTGGCTACCATCTGTGAA | 102 | 62 |
| 1 | HOXD3 (NM010468) | CCATAAATCAGCCGCAAGGA GGATGGGTCGAGGACTTACCTTAG | 112 | 63 |
| 152 | GPI (NM008155) | TGGACGGCAAAGATGTGATG CGATGTTGATGATGTCGGTGA | 129 | 63 |
| 152 | VEGF (NM009505) | ATCGGGATCAAACCTCACCA CCGCTCTGAACAAGGCTCAC | 129 | 63 |
| 136 | TNFR1 (NM011609) | CTGCTCTACGAATCACTCTGCTC ACAGCATACAGAATCGCAAGGTC | 113 | 62 |
| 151 | HNF4 (NM008261) | CCAAGTACATCCCGGCCCTTC CTAGGAGCAGCACGTCCTTAAAC | 132 | 62 |
| 151 | FGF2 (NM008006) | GGCTGCTGGCTTCTAAGTGTG ACTGCCAGTTCGTTTCAGTG | 129 | 62 |

2. Materials and methods

2.1. Experimental protocol (GFP/CCl₄ model)

We developed a new *in vivo* model in which we could monitor the plasticity of BMCs into hepatocytes [12,16]. The mouse line C57BL/6/Tg14 (act-EGFP) OsbY01 was a kind gift from Dr. Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [14]. C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). We injected 0.5 ml/kg body weight of CCl₄ into C57BL/6 mice at 6 weeks of age via the peritoneum twice a week for 4 weeks to induce persistent liver damage. At this time, the condition of recipient mice was liver cirrhosis. One day after 4 weeks of CCl₄ injection, 1×10^5 GFP-positive BMCs were injected slowly using a 31 G needle and Hamilton syringe via the tail vein. The mice that were injected with CCl₄ only were used as the control group. After BMC transplantation, the same dose of CCl₄ was injected twice a week. Individual mice were killed at 18 h after initial CCl₄ injection (2 days after BMC transplantation) and once a week after BMC transplantation for 4 weeks. All processes including surgical steps confirmed to the guidance of Yamaguchi University for animal and recombinant DNA experiments.

2.2. RNA preparation and microarray analysis

In both the BMC transplantation and control groups, the liver was excised 2 days and 1, 2, 3, and 4 weeks after transplantation. The mice were killed by cervical dislocation. The whole liver was removed and immediately frozen in liquid nitrogen. Liver samples were pooled at least two from whole liver of both mice groups (BMC transplantation and control CCl₄ damage at each point). Total RNA was isolated from pooled liver samples using an Atlas Glass Total RNA Isolation Kit (Clontech, Palo Alto, CA) [21]. Single strands of cDNA were synthesized using the primer mix, dNTP, aminoacryl dUTP, and MMLV-RT using an Atlas Glass Fluorescent Labeling Kit (Clontech). The synthesized cDNA probes were coupled to monoreactive Cy3 for fluorescent labeling. Probes were prepared in the same manner for the control group (no BMC transplantation) and BMC transplantation group at the same time. The DNA microarray analysis was conducted using an Atlas Glass Mouse 1.0K Microarray System (Clontech) [22]. The above-mentioned cDNA probes were hybridized to a DNA chip composed of about 1100 DNA fragments by incubating the chip overnight at 50 °C with the probe. After incubation, the chip was washed using GlassHyb Wash Solution, RNase water and 20× SSC, rinsed with distilled water and then air dried. The signal intensity of each gene was measured using a fluorescent scanner (Axon Instruments, CA). The spot intensity of expression of each gene was assessed using the ArrayGauge System (Fuji Film, Tokyo, Japan). The raw data of the spot intensity were used for SOM analysis (All raw data of microarray are available at <http://liver-project.med.yamaguchi-u.ac.jp/research/>). We performed several analyses to obtain representative data.

2.3. SOM analysis for microarray

The microarray analysis showed that, of the 1100 genes on the DNA chips, although the expression of some genes was too small for further analysis, the expression data recorded of the remaining 1018 genes were sufficient for SOM data analysis. At each of five sampling times, i.e., 2 days and each week for 4 weeks, expression levels of 1018 genes for both control CCl₄ damage and BMC transplantation group were measured, respectively. To extract the genes that are differentially expressed before and after BMC transplantation, we defined the following equation (Fig. 1):

$$f_i(t) = \log_{10} \frac{y_i(t)}{x_i(t)}, \quad t = 1, 2, 3, 4, 5, \quad i = 1, 2, \dots, 1018 \quad (1)$$

where $x_i(t)$ is the expression level of the control CCl₄ alone sample (CCl₄ alone without BMC transplantation) and $y_i(t)$ is the expression level of BMC transplantation sample for gene i (1, 2, ..., 1018) at sampling point t , respectively. The term $f_i(t)$ represents the expression level of GFP/CCl₄ group normalized by the control group. If $y_i(t) = x_i(t)$, at that the value of $f_i(t)$, i.e., $\log_{10} 1$, was zero. By using this, we succeeded in extracting the change of gene expression by BMC transplantation. Point t shows time. $t = 2d, 1w, 2w, 3w, 4w$ showed 1, 2, 3, 4, and 5, respectively. To extract the change of gene expression with time, we followed the change of the values of

$f_i(t+1) - f_i(t)$ with the increase in t . Using $f_i(t)$, we defined the 4-dimensional vector $F_i = [F_i(1), F_i(2), F_i(3), F_i(4)]^T$ for gene i , where

$$F_i(1) = f_i(2) - f_i(1)$$

$$F_i(2) = f_i(3) - f_i(2)$$

$$F_i(3) = f_i(4) - f_i(3)$$

$$F_i(4) = f_i(5) - f_i(4)$$

All genes were described and then used as input patterns for SOM analysis. SOM was performed using the SOM toolbox in MATLAB (The Mathworks Inc., Natick, MA; and <http://www.cis.lut.fi/projects/somtoolbox/>).

Each element of these vectors [$F_i(i = 1, 2, 3, \dots, 1018)$] represents a chronological change of gene expression after BMC transplantation in GFP/CCl₄ model.

2.4. Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from the whole liver of both mice groups (BMC transplantation and control CCl₄ damage ($n = 2$, each group) using Isogen Total-RNA isolation kit (Nippon Gene Co., Ltd., Tokyo, Japan) at each of five sampling times, i.e., 2 days and each week for 4 weeks. These samples were obtained by independent experiments from microarray analysis. RT step was performed using SYBR RT-PCR kit (Takara Co., Tokyo, Japan).

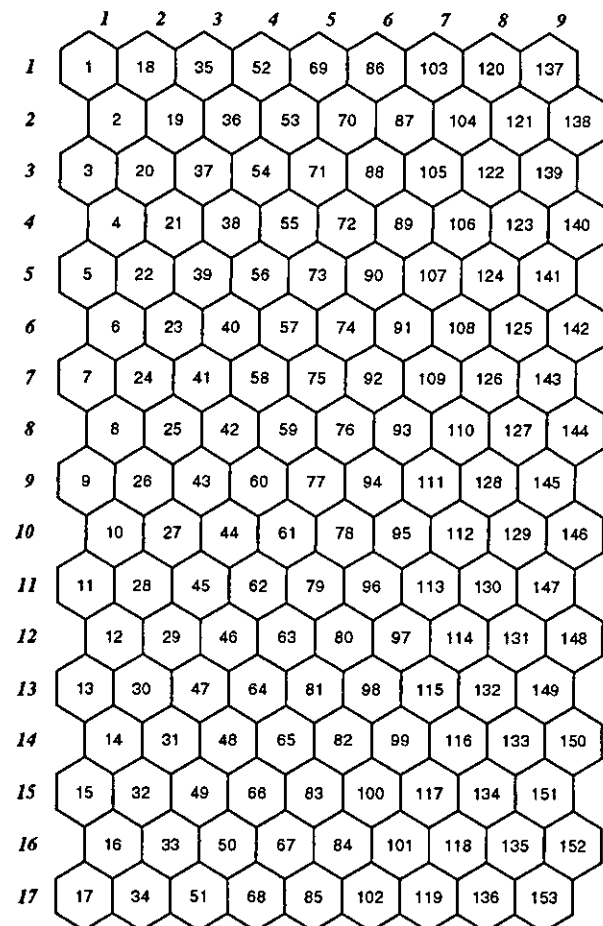


Fig. 2. The 1018 genes analyzed using microarray were divided into 153 clusters and arranged in a 17 × 9 matrix (height × width) of 153 hexagons (all raw data of microarray are available at <http://liver-project.med.yamaguchi-u.ac.jp/research/>). The number of genes varied among the clusters. Clusters with similar elements were arranged close to each other in the matrix.

Two μl of cDNA solution (100 ng of initial RNA) was amplified in 20 μl of reaction mixture containing 5 pmol of forward and reverse primer. PCR was performed for a total of 45 cycles, each of 95 $^{\circ}\text{C}$ for 5 s and 60 $^{\circ}\text{C}$ for 20 s [23]. We selected 13 genes to further clarify the difference expression pattern of each gene. c-kit, fibroblast growth factor (FGF)-6, matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-2, Hepatocyte growth factor (HGF), Numbl-like (NumbL) and homeobox (HOX) - D3, Glucose-6-phosphatase isomerase (GPI), vascular endothelial growth factor (VEGF), tumor necrosis factor receptor (TNFR)-1, hepatocyte nuclear factor (HNF)-4 and FGF-2 were selected. The primer used in this study is shown in Table 1. The relative ratio of each gene expression was determined referring with the mean expression level of control house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 S ribosomal RNA expression.

2.5. SOM analysis compared between RT-PCR and microarray

To validate the results of SOM analysis depend on microarray, we compared SOM analysis between microarray and RT-PCR. We used the same equation and performed SOM analysis (Fig. 1) based on both the data of RT-PCR and microarray.

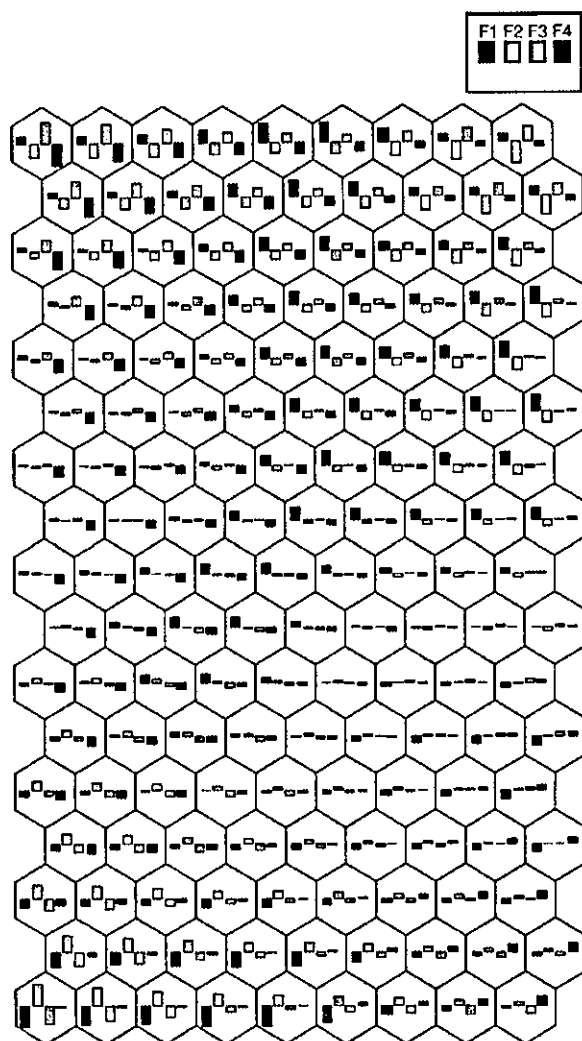


Fig. 3. The median value for gene expression for F_1 , F_2 , F_3 , and F_4 in each cluster is presented as a bar chart. As a reference, in cluster 1, the median value in F_1 and F_3 was increased, while that in F_2 and F_4 was decreased.

3. Results

The 1018 genes that could be analyzed by the DNA chips were classified into 153 clusters by SOM (Fig. 2). Genes in the same cluster showed similar gene expression pattern during the process of BMC trans-differentiation. On the SOM matrix, clusters with similar vector F_i elements (F_1 – F_4) were arranged in close proximity to each other. Therefore, adjacent clusters on the matrix exhibited similar chronological changes in gene expression profiles during the process of plasticity of BMC into hepatocyte. Fig. 3 shows bar charts that represent the median value of gene expression for each cluster in F_1 , F_2 , F_3 , and F_4 . By analyzing each element (F_1 – F_4) of vector F_i , the clusters were color coded to aid visualization of the SOM data (Fig. 4). For example, in the F_1 output, clusters 69, 70, and 86 containing upregulated genes in F_1 were colored dark brown. On the other hand, clusters with downregulated genes in F_1 were colored dark blue. The color bar on the right-hand side of the figure indicates the degree of gene expression from F_1 to F_4 , and a value of 0 indicates that there was little transient change in gene expression between the BMC transplantation and control CCl_4 injection groups. The following clusters exhibited marked changes in transient gene expression: in F_1 , clusters 69, 70, 86, 92, 125, 140, 141, 142, and 143; in F_2 , clusters 13, 14, 15, 16, 17, 32, 33, and 34; in F_3 , clusters 1, 2, 18, and 137; and in F_4 , clusters 118, 119, 133, 134, 135, 136, 148, 149, 150, 151, 152, and 153. To validate the data of SOM analysis based on microarray analysis, we performed SOM

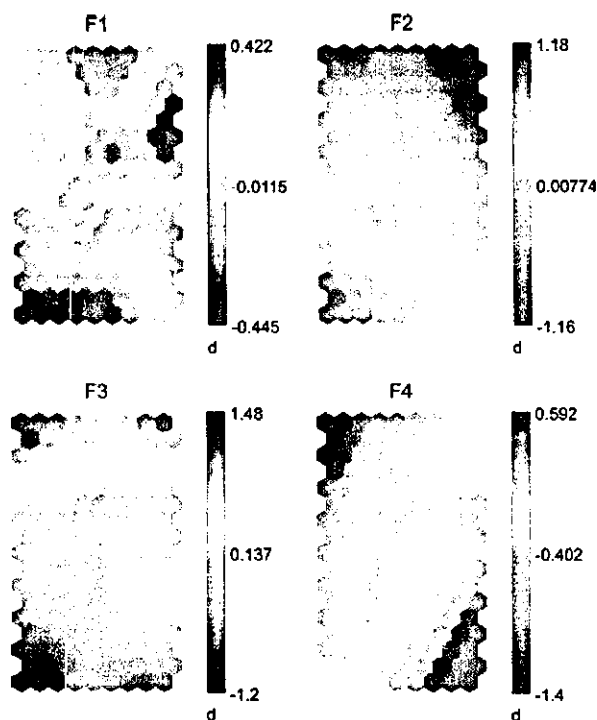


Fig. 4. The clusters were color-coded based on the median values for gene expression at the F_1 , F_2 , F_3 , and F_4 time periods. The color bar on the right hand side indicates the values in each period; dark brown for upregulated and dark blue for downregulated. A value of 0 indicates that there was little chronological change in gene expression between the transplantation and control groups.

analysis based on the data of RT-PCR for selected 13 genes using the same equation (Figs. 5A and B). Table 2 shows the list of genes in each cluster.

4. Discussion

By using specific equation to extract change of gene expression after BMC transplantation, we found that there were dramatic changes for both gene expression and distribution of gene clusters after BMC transplantation in GFP/CCL₄ model (Figs. 3 and 4). 1018 genes were classified into 153 patterns of change of gene expression using SOM analysis. These results might show that many genes had important reciprocal roles during the process of differentiation of BMC into albumin positive hepatocyte. To validate the SOM analysis based on microarray analysis, we performed SOM analysis based on selected 13 gene expressions analyzed by RT-PCR independently. *c-kit*, *FGF6*, *MMP2*, *MMP9*, and *TIMP2* were selected from serious clusters at *F1* periods (clusters 86, 92, and 125). *HGF* was selected from *F2* periods (cluster 13). *NumbL* and *HOXD3* were selected from *F3* (cluster 1). *GPI*, *VEGF*, *TNFR1*, *HNF4*, and *FGF2* were selected from *F4* (clusters

136, 151, and 152). As shown in Fig. 5, we found the similar position of these selected genes. This means that the change of gene expression from microarray analysis is similar to that from RT-PCR analysis. These results showed the consistency of SOM analysis based on microarray using specific equation.

Cluster with color deeper than dark orange (69, 70, 125, 141, 142, and 143) showed the dramatic change in *F1* period (Fig. 4). The *c-kit* gene, which was present in cluster 86, encodes a stem cell factor receptor which is related with rat hepatic stem cell, oval cell, activation [24]. *FGF-6* was also extracted in cluster 86. *FGF* was known to have an important role of hepatocyte proliferation and liver development [25]. To focus on the change of *F1* to *F4* in cluster 86, we found that genes in cluster 86 were upregulated soon after BMC transplantation suggesting that the expression of these genes changes dynamically and might have an important role in the early stage of plasticity of BMC (Fig. 3). In cluster 125, genes involved in the regulation of liver fibrosis such as *MMP2* and *MMP9* were pointed out. These results were consistent with liver fibrosis recovered by BMC transplantation. *MMP9* has been reported to facilitate the induction of hematopoietic cells from the marrow via the kit signal transduction pathway [26]. This result might suggest that ECM might be important for the

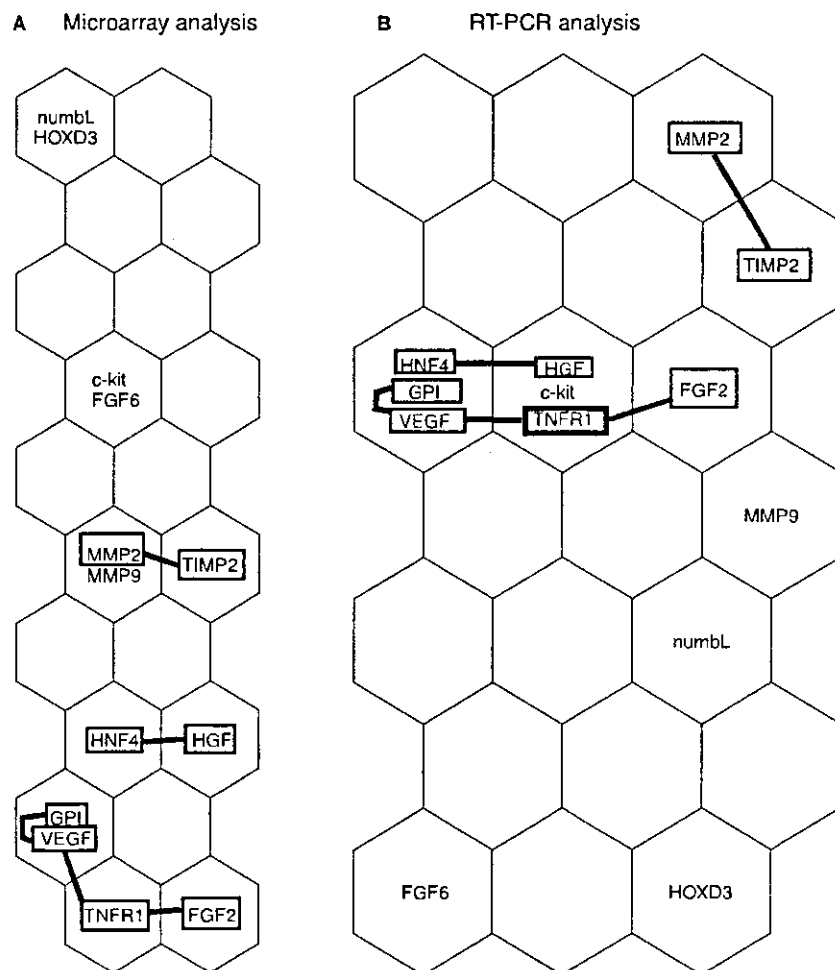


Fig. 5. For the genes, the data of microarray (A) and RT-PCR (B) were classified into clusters by SOM, respectively. Clusters with similar elements were arranged close to each other in the matrix.

Table 2
Selected gene grouped to clusters

| Cluster No. | GenBank ID | Gene description | |
|---|-------------------------------|---|---|
| Clusters with remarkable variation of gene expression in F1 | | | |
| 86 | U52951 | Enhancer of zeste homolog 2 (EZH2); ENX1H | |
| | X06368 X68932 | Macrophage colony-stimulating factor 1 receptor (CSF1R) o-fms proto-oncogene | |
| | Y00864 NM02099 | c-kit proto-oncogene | |
| | U14173 | ski proto-oncogene | |
| | M92415 | Fibroblast growth factor 6 (FGF6) | |
| 69 | X07439 | Homeobox protein 3.1 (HOX3.1) | |
| | X57487 | Paired box protein 8 (PAX8) | |
| | U43788 | POU domain class 2-associating factor 1 (POU2AF1); OCT-binding factor 1 (OBF1); BOB1; OCA-B | |
| | M84819 | Retinoic acid receptor- γ (RXR- γ ; RXRG) | |
| | AF015848 | E2F transcription factor 3 (E2F3) | |
| | Z22649 | mpl proto-oncogene; thrombopoietin receptor | |
| | L27105 | MOESIN-ezrin-radixin-like protein (MERLIN); schwannomin (SCH); neurofibromatosis 2(NF2) | |
| | U51196 | EB1 APC-binding protein | |
| | Y00671 | met proto-oncogene | |
| | Z12604 | Matrix metalloproteinase 11 (MMP11); stromelysin 3 (STMY3) | |
| 70 | D90156 | Myogenin (MYOG); myoD1-related protein | |
| | U51037 | CCCTC-binding factor (CTCF) | |
| | U06119 | Cathepsin H | |
| 142 | J04946 | Angiotensin-converting enzyme (ACE); dipeptidyl carboxypeptidase I (DCP1); kinase II | |
| | U10551 | Gem induced immediate early protein | |
| | L34290 | Transducin β -5 subunit GTP-binding protein G(i)/G(s)/G(t) β subunit 3 | |
| | M96653 | Adenylate cyclase 6 | |
| | Z71173 | Inositol 1,4,5-triphosphate receptor 2 | |
| | L76946 | Phosphodiesterase 1C | |
| | U12919 | Adenylate cyclase type VII (AIP pyrophosphate-lyase) (adenylyl cyclase) (KTAA0037) | |
| | D31788 | BP3 alloantigen | |
| | AA000715 | S100 calcium-binding protein A1; S-100 protein α chain | |
| | U97327 | Calcylin binding protein | |
| | U73004 | Antileukoproteinase 1 (ALP1); secretory leukocyte protease inhibitor | |
| | 140 | U58992 | Mothers against decapentaplegic homolog 1 (MADH1; mSMAD1); TGF- β signaling protein 1 |
| | | U36203 | snoN: ski-related oncogene |
| D17571 | | NADPH-cytochrome P450 reductase (CPR); POR | |
| U68058 | | Secreted frizzled-related protein 3 (SFRP3; mFIZ); frizzled | |
| U77638 | | Mothers against dpp homolog 5 (SMAD5; MADH5) | |
| M33385 | | Neurotrophic tyrosine kinase receptor type 2 (NTRK); tyrosine kinase receptor B (TRKB) | |
| X57277 | | ras-related C3 botulinum substrate 1 (RAC1) | |
| M21531 | | Calbindin-28K: calbindin 1 (CALB1) | |
| U53142 | | Nitric oxide synthase 3, endothelial cell | |
| U37775 | | Tuberin: TSC2 (tuberous sclerosis 2 protein) | |
| J05261 | | Lysosomal protective protein; cathepsin A; carboxypeptidase C (CPC); MO54 | |
| M81591 | | Membrane metallo endopeptidase | |
| M14222 | | Cathepsin B (CTSB) | |
| X15475 | | Peripherin (PRPH) | |
| 141 | | U61969 | Wingless-related MMTV integration site 10a protein (WNT10A) |
| | X76292 | Desert hedgehog homolog (DHH); HHG3 | |
| | X67962 | Interleukin 7 (IL7) | |
| | X66196 | Recoverin (RCV1; RCVRN); cancer-associated retinopathy protein (CAR protein), 23-kDa photoreceptor cell-specific protein | |
| | U57311 | 14-3-3 protein η ; protein kinase C inhibitor protein 1 (KCIP1); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein η polypeptide (YWHAH) | |
| | X16490 | Macrophage plasminogen activator inhibitor 2 (PAI2; PLANH2) | |
| | X05211 | Laminin γ 1 subunit (LAMC1); laminin B2 subunit | |
| 125 | U03421 | Interleukin 11 (IL11) | |
| | M84324 | Matrix metalloproteinase 2 (MMP2) | |
| | NM008610 | | |
| | X72795 | Matrix metalloproteinase 9 (MMP9) | |
| | NM013599 | | |
| M59470 | Cystatin C; cystatin 3 (CST3) | | |

(continued on next page)

Table 2 (continued)

| Cluster No. | GenBank ID | Gene description | |
|---|------------|---|--|
| 143 | U94350 | Radical fringe homolog (RFNG) | |
| | U37720 | Cell division cycle 42 homolog (CDC42); 25-kDa GTP-binding protein (G25K) | |
| | X73985 | Calbindin 2 (CALB2); calretinin | |
| | X61432 | Calmodulin (CALM; CAM) | |
| | U48853 | ork-associated substrate (CRKAS; CAS) | |
| | U24680 | Dishevelled 2 homolog (DVL2) | |
| | D00611 | Basic immunoglobulin superfamily (BASIGIN; BSG); membrane glycoprotein 42 (GP42), neurothelin; CD147 antigen | |
| | M28730 | Tubulin β 4 (TUBB4) | |
| | Z21848 | DNA polymerase δ catalytic subunit (POLD1) | |
| | 92 | U37331 | T-box protein 6 (TBX6) |
| M61909 | | relA proto-oncogene; NF- κ -B transcription factor p65 subunit (NF- κ B p65) | |
| U18542 | | Calcitonin receptor 1b | |
| U17985 | | Cannabinoid receptor 1 (CNR1; CBR), brain cannabinoid receptor | |
| U41285 | | Segment polarity protein dishevelled homolog 3 (DVL3), DSH homolog 3 | |
| X62622 | | Tissue inhibitor of metalloproteinase 2 (TIMP2) | |
| NM011594 | | | |
| L07803 | | Thrombospondin 2 (THBS2; TSP2) | |
| X53929 | | Decorin (DCN); bone proteoglycan II (PG-S2), PG40 | |
| X61435 | | Neuronal kinesin heavy chain (NKHC); KIF5C | |
| D10061 | | DNA topoisomerase I (<i>Top1</i>) | |
| D28492 | | Caspase 2 (CASP2); NEDD2 protein; ICH1 cysteine protease | |
| X66323 | | X-ray repair-complementing defective repair in Chinese hamster cells 5 (XRCC5) | |
| Clusters with remarkable variation of gene expression in F2 | | | |
| 17 | | AF017085 | BAP-135 homolog; DIWSIT; general transcription factor II-1 (GTF2I) |
| | U77969 | Neuronal PAS domain protein 2 | |
| | X97817 | Semaphorin F (SEMAF) | |
| | M84487 | Vascular cell adhesion molecule 1 (VCAM1) | |
| | M59378 | Tumor necrosis factor receptor superfamily member 1B2 (TNFRSF1B2); tumor necrosis factor receptor 2 (TNFR2) | |
| | U25995 | Receptor (TNFRSF)-interacting serine-threonine protein kinase 1 (RIPK1; RIP) | |
| | L22472 | BCL2-associated X protein membrane isoform alpha (BAX-alpha) | |
| | L33406 | Uromodulin | |
| | AF007268 | Fibroblast growth factor 15 (FGF15) | |
| | AF030433 | Dickkopf homolog 1 (mDKK1) | |
| | AF031896 | Cerberus-related protein1 (CERR1) | |
| | X58995 | Calcium/calmodulin-dependent protein kinase IV catalytic subunit (CAM kinase-CR; CAMKIV; | |
| | 34 | D32132 | Hairy and enhancer of split protein 5 (HES5) |
| Z93947 | | Semaphorin H (SEMAH) | |
| U69535 | | Semaphorin J (SEMAJ) | |
| X97818 | | Semaphorin G (SEMAG); SEMA5B | |
| U25416 | | Tumor necrosis factor receptor superfamily member 8 (TNFRSF8); CD30L receptor | |
| U39643 | | fas-associated factor 1 (FAF1) | |
| Z22703 | | Fibroblast growth factor 7 (FGF7) | |
| X63615 | | Calcium/calmodulin-dependent protein kinase type II β subunit (CAM-kinase II β ; CAMK-II β) | |
| U43187 | | Mitogen-activated protein kinase kinase kinase 3 (MAPKKK3; MAP3K3; MAPK/ERK kinase kinase 3 (MEK kinase 3; MEKK3) | |
| L35236 | | Mitogen-activated protein kinase 10 (MAP kinase 10 MAPK10; PRKM10); MAP kinase p49 3F12; Stress-activated o-jun N-terminal kinase 3 (JNK3); SAPK/ERK kinase 2 (SERK2) | |
| U03856 | | Receptor-type protein tyrosine phosphatase (PTPRCAP); C polypeptide-associated protein; CD45-Associated protein (C045-AP), LSM | |
| 16 | L20331 | Adenosine A3 receptor | |
| | L41145 | Bone morphogenetic protein 5 (BMP5) | |
| | AB006787 | Mitogen-activated protein kinase kinase kinase 5 (MAPKKK5; MAP3K5); MAPK/ERK kinase kinase 5 (MEKK5); apoptosis signal-regulating kinase 1 (ASK1) | |
| | U92456 | Serine/alanine-rich protein specific kinase 2 (SRPK2), WW domain binding protein 6 (WBP6) | |
| 15 | X81579 | Insulin-like growth factor-binding protein 1 (IGF-binding protein 1; K3FBP1) | |
| | U60530 | Mothers against decapentaplegic homolog 2 (MADH2; mSMAD2) | |
| | AB005141 | Klotho protein (KL) | |
| | U77039 | Four and a half LM domains 1 (FLH1); KyoT | |
| 51 | Z32675 | Hairless protein (HR) | |
| | L10075 | DNA-binding protein SMBP2 | |
| | S56660 | Retinoic acid receptor β (RAR- β ; RARB); nuclear receptor subfamily 1 group B member 2 | |

Table 2 (continued)

| Cluster No. | GenBank ID | Gene description |
|--|--|---|
| 32 | X57413 | Transforming growth factor β 2 (TGF- β 2; TGF β 2) |
| | M20473 | cAMP-dependent protein kinase type I β regulatory chain (PRKAR1B) |
| | Y00703 | Guanine nucleotide-binding protein alpha stimulating activity polypeptide (GNAS) |
| | Y07836 | Stimulated by retinoic acid protein 14 (STRA14); STRA13; E47 interaction protein 1 (EIP1) |
| | X85993 | Collapsin 1; semaphorin IIIA (SEMA3A), SEMAD |
| | L28177 | Growth arrest and DNA damage-inducible protein (GADD45), DNA damage-inducible transcript 1 |
| | L33768 | Janus tyrosine-protein kinase 3 (JAK3) |
| | U07617 | Growth factor receptor-bound protein 2 (GRB2); ASH protein |
| | L02526 | Mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1; MAPKK1; MAP2K1; PRKMK1); MAPK/ERK kinase 1 (MEK1) |
| | U66058 | DNA ligase III; polydeoxyribonucleotide synthase (ATP) (DNL3) |
| 33 | L28819 | Involucrin (IVL) |
| | X97052 | Mitogen-activated protein kinase kinase 6 (MAP kinase kinase 6; MAPKK6; MAP2K6; PRKMK6); MAPK/ERK kinase 6 (MEK6); SAPKK3 |
| | L09562 | Protein-tyrosine phosphatase γ (R-PTP- γ ; PTPRG) |
| Z32767 | DNA damage repair & recombination protein 52 homolog (RAD52) | |
| 14 | X81466 | Ephrin type A receptor 7 (ephrin receptor A7; EPHA7), embryonic brain receptor tyrosine kinase (EBK); developmental kinase 1 (mDK1) |
| | U37522 | Tumor necrosis factor superfamily member 10 (TNFSF10); TNF-related apoptosis inducing ligand |
| | D38258 | Fibroblast growth factor 9 |
| | X77113 | Growth differentiation factor 9 (GDF9) |
| | X06381 | Leukemia inhibitory factor (LIF); cholinergic differentiation factor |
| | Z22532 | Syndecan 1 (SYND1) |
| | M75716 | Alpha-1-antitrypsin, 1-2 (AAT2), serine proteinase inhibitor 1-2 (SPI1-2); alpha 1 protease inhibitor 2; alpha-1- antiproteinase |
| | M64292 | Anti-proliferative B-cell translocation gene 2 (BTG2); NGF- inducible protein TIS21 |
| | L23971 | Fragile X mental retardation syndrome 1 homolog (FMR1; FMRP) |
| | 13 | U95610 |
| U05252 | | Special AT-rich sequence-binding protein 1 (SATB1) |
| U70017 | | Cyclin-D binding Myb-like protein (hDMPI) |
| AF001287 | | Neural cell adhesion molecule 2 (NCAM2), olfactory axon cell adhesion molecule (OCAM) |
| J04806 | | Osteopontin (OP); bone sialoprotein 1; minopontin; early T-lymphocyte activation 1 protein (ETA1); secreted phosphoprotein 1 (SPP1), calcium oxalate crystal growth inhibitor protein |
| X83930 | | Cadherin 5 (CDH5); vascular epithelial cadherin (VE-cadherin) |
| AF003747 | | Zinc transporter 4 (ZNT4) |
| M16472 | | Myelin proteolipid protein (PLP), lipophilin; DM20 |
| XI5830 | | 7B2 neuroendocrine protein: secretogranin V (SGV; SCG5) |
| X72307 NM010427 | | Hepatocyte growth factor (HGF) |
| D84372 | Non-receptor type II protein cyrosome, phosphocytosome phosphrase | |
| M38700 | ATP-dependent DNA helicase II 70-kDa subunit 70-kDa thyroid autoantigen; lupus Ku autoantigen protein p70 CTC box-binding factor 75-kDa subunit (CTCBF; CTC75) | |
| cluster with remarkable variation of gene expression in F3 | UB6441 NM010950 | numfaiike (numbL; m-nbl) |
| | M33158 | CD3 antigen zeta (CD3Z) |
| | X04648 | Low-affinity IgG Fc receptor II β (FCGR2B) |
| | D49658 | LIM-homeodomain protein L3; LHX8 |
| | L12705 | Engrailed protein (En-2) homolog |
| | D49474 | SRY-box containing gene 17 (SOX17) |
| | X73573 | Homeobox protein D3 (HOXD3) |
| | NM010468 | |
| | U62522 | Sp4 zinc finger transcription factor |
| | U25096 | Lung Kruppel like factor (LKLF) |
| X90329 | Lbx 1 transcription factor | |
| X61753 | Heat shock factor 1 | |
| U53925 | Transcription factor C1 | |
| U97076 | FLICE-like inhibitory protein long form (FLIP-L) | |
| 18 | M55S12 | WT1; Wilms tumor protein; tumor suppressor |
| | M16449 | Myeloblastosis proto-oncogene (MYB) |
| | X13945 | Lung carcinoma myc-related oncogene 1 (L-myc; mycL1) |
| | M26391 | Retinoblastoma-associated protein 1 (RB1); phosphoprotein 105 (PP105) |
| | U65594 | Breast cancer type 2 susceptibility protein (BRCA2) |
| | U04807 | FMS-like tyrosine kinase 3 ligand (FLT3L) |
| | M34563 | T-ceB-specific surface glycoprotein CD28 |

(continued on next page)

Table 2 (continued)

| Cluster No. | GenBank ID | Gene description |
|---|---|---|
| 2 | M32240 M63801 AF013282 U63386 J05154 | Peripheral myelin protein 22 (PMP22); CD25 antigen; SR13 myelin protein Gap junction alpha 1 protein (GJA1), connexin 43 (CXN43; CX43) T-box protein 15 (TBX15); TBX14; TBX8 Early development regulator 1 (EDRI); pdyhomeotic 1 homolog (mPHI) Lecithin cholesterol acyltransferase (LCAT); phosphatidylcholine sterot acyltransferase; phospholipid cholesterol acyltransferase |
| 137 | M55171 D50311 X14943 U12570 V00727 U36799 S59388 Z31683 S53216 AF039601 X06203 | Rhodopsin (RHO), opsin (mOPS) Myocyte enhancer factor 2B (MEF2B) Contactin 1 (CNTN1); F3 neuronal cell adhesion molecule (F3CAM) von Hippel-Lindau syndrome homolog (VHLH) fos proto-oncogene retinoblastoma- like protein 2 (RSL2); retinoblastoma-related protein PRB2/p130 Erythropoietin receptor (EPOR) activin A receptor type 1B Tyrosine-protein kinase ryk; kinase vik; nyk-R TGF-beta receptor type IB (betaglycan); candidate tumor suppressor gene Interleukin 8 (IL6) |
| Clusters with remarkable variation of gene expression in F4 | | |
| 152 | L12140 M98502 S79463 X91144 U04294 M14220 NM008155 M95200 NM009505 M30643 U51866 U17112 U67916 U49739 | Groucho gene-related protein (GRG); amino enhancer of split protein (AES) Zinc finger protein 46 Semaphorin I (SEMAI) P-selectin glycoprotein ligand 1 (PSGL1; SELPLG; SELP1) Electrocardiographic QT syndrome 2 potassium channel subunit Glucose-6-phosphate isomerase (GPI) Vascular endothelial growth factor (VEGF) Heparin-finding growth factor 5 (HBGF5); fibroblast growth factor 5 (FGF5) Casein kinase II alpha 1 related sequence 4 (CSNK2A1-RS4) Dentin sialophosphoprotein (DSPP) Unconventional myosin VI |
| 153 | S663B5 X85994 U28724 | CREB-binding protein Semaphorin IIIC (SEMA3C); SEMAE Postmeiotic segregation increased 2 homolog (PMS2) |
| 135 | X12875 L24755 X83106 U34960 D86726 | Neural cell adhesion molecule LI (N-CAM LI; LiCAM; CAMLI) Bone morphogenetic protein 1 (BMP1) MAX dimerization protein (MAD) Transducin beta-2 subunit MCM6 DMA replication licensing factor (P105MCM) |
| 136 | D31967 U59496 X85992 X07640 X57796 NM011609 X53798 X78850 U43144 X83536 Y13602 | Jumonji protein Hypoxia inducible factor 1 alpha subunit (HIF1-alpha; HIF1A); ARNT-interacting protein Somaphorin C (SEMAC) Cell surface glycoprotein MAC-1 alpha subunit; CR-3 alpha subunit; GD11B antigen; leukocyte adhesion receptor MO1; integrin alpha-M (ITGAM) Tumor necrosis factor receptor 1 (TNFR1) Small inducible cytokine subfamily B member 2 (SCYB2); macrophage inflammatory protein 2 (MIP2) Mitogen activated kinase-activated protein kinase 2 (MAPKAP kinase 2; MAPKAPK2); 60-kDa ribosomal protein S6 kinase polypeptide 1 (RPS6KC1) Phospholipase C beta 3 (PLC-beta 3; PLCB3) Matrix metalloproteinase 14 (MMP14); membrane-type matrix matatoproteinase 1 (MTMMP1) Filensin, beaded filament structural protein in lens 1 (BFSP1) |
| 150 | AF033011 D63644 S70632 S70756 S70629 S81932 X75330 U89487 U89489 U36340 U42554 U33626 | distal-less homeobox protein 5 (DLX5) Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) T-cell leukemia homeobox 1 (TLX1); homeobox protein 11 (HOX11) distal-less homeobox protein 3 (DLX3) Drosophila NK5 transcription factor-related locus 1 (NKX-5.1), H6 homeobox protein 3 (HMx3) LIM homeobox protein cofactor 1A (CUM1A); CUM1B; LIM domain-binding protein 3 (LDB3) CACCC-box-binding protein basic Kruppel like factor (BKLF); Kruppel-like factor 3 (KLF3) Single-minded 2 (SIM2) Promyelocytic leukemia gene (PML) |

Table 2 (continued)

| Cluster No. | GenBank ID | Gene description |
|-------------|------------|--|
| | X56135 | Prothymosin alpha (PTMA) |
| | U43512 | Dystroglycan 1 |
| | M13071 | A-raf proto-oncogene |
| | M36829 | Heat shock 84-kDa protein 1 (HSP84-1); HSP90 |
| | M89802 | Wingless-related MMTV integration site 7b protein (WNT7B) |
| | M30903 | B-lymphocyte kinase (BLX) |
| | U43298 | Laminin β 3 subunit (LAMB3); kalinin B1 subunit |
| 151 | D29015 | Hepatic nuclear factor 4-alpha (HNF4-alpha) |
| | NM008281 | |
| | M31042 | Immediate early response protein 2 (IER2); T-lymphocyte activated protein; cycloheximide-induced Protein 1 (CHX1) |
| | U43900 | STAM; signal transducing adaptor molecule |
| | M13177 | Transforming growth factor β 1 (TGF- β 1; TGFB1) |
| | M30644 | Fibroblast growth factor 2 (FGF2) |
| | NM008006 | |
| | M76601 | Cardiac myosin heavy subunit alpha isoform (MYH6; MYHCA) |
| 148 | AB009453 | Transcription factor 21 (TCF21); basic helix-loop-helix factor COR1; POD1 |
| | S71659 | LIM homeobox protein 4 (LHX4); GSH4 |
| | S79041 | Genomic screened homeobox protein 2 (GSH2) |
| | U58533 | est2 repressor factor (ERF) |
| | J03770 | Homeobox protein D4 (HOXD4); HOX4.2; HOX5.1 |
| | X59252 | Homeobox protein 8 (HOX8) |
| | J03168 | Interferon regulatory factor 2 (IRF2) |
| | S68108 | Brahma-related protein 1 (BRG1); swi/snf-related matrix-associated actin dependent regulator of chromatin subfamily a member 4 (SMARCA4) |
| | D78382 | Transducer of erbB2 (TROB; TOB) |
| | X59421 | Fli-1 ets-related proto-oncogene |
| | M62860 | Myelin protein zero |
| | D31942 | Oncostatin M (OSM) |
| | M29464 | Platelet-derived growth factor A subunit (PDGFA; POGF1) |
| | M69042 | PKC- δ ; protein kinase C δ type |
| | U29539 | Retinoic acid-inducible E3 protein; stimulated by retinoic acid 13 (STRA13), hematopoietic-specific Protein E3; orfB |
| | U26967 | Cordon-bleu protein (COBL) |
| 149 | L38248 | LIM homeobox protein 3 (LHX3; UM3) |
| | X56230 | Octamer-binding transcription factor 1 (OCT1; OTF1); NF-A1; POU domain class 2 transcription factor 1 (POU2F1) |
| | X13721 | Homeobox protein 2.4 (HOX2.4) |
| | L04662 | γ -Aminobutyric acid transporter 4 (GABA-A transporter 4; GABT4) |
| | U59746 | B-cell lymphoma protein W (BCLW); BCL2-like protein 2 (BCL2L2) |
| | M16819 | Lymphotoxin alpha (LTA), tumor necrosis factor β (TNF- β ; TNFB) |
| | U12147 | Laminin alpha 2 subunit (LAMA2), dystrophin muscularis protein (DV); merosin heavy chain; laminin M subunit |
| | U22421 | Leptin (LEP); obese factor (OB) |
| 133 | D10329 | CD7 antigen |
| | D86603 | btb and cnc homolog 1 (BACH1) |
| | U36384 | Dermis expressed 1 protein (DERM01) |
| | M58633 | P58/GTA; galactosyl transferase associated protein kinase (cdc2-related protein kinase) |
| | D83698 | Activator of apoptosis harakiri (HRK); neuronal death protein 5 (DP5); BID3 |
| | L08235 | Clusterin (CLU); clustrin; apolipoprotein J (APOJ); sulfated glycoprotein 2 (SGP2; mSGP2) |
| | X07414 | DNA excision repair protein ERCC1 |
| 134 | U61155 | LIM homeobox protein 2 (LIM2); LHX5 |
| | U20553 | p57kip2; cdk-inhibitor kip2 (cyclin-dependent kinase inhibitor 1 B); member of the p21CIP1 Cdk inhibitor family; candidate tumor suppressor gene |
| | U05671 | Adenosine A ₁ receptor (ADORA1) |
| | L37663 | Acetylcholine receptor alpha 7 neural |
| | M97017 | Bone morphogenetic protein 8A (BMP8A), osteogenic protein 2 (OPS) |
| | AF027503 | guanylate kinase membrane-associated inverted protein 1 (GUKMI1; MAGI-1) |
| | M84817 | Retinoid X receptor alpha (RXR- α); RXRA |
| | J03520 | Tissue plasminogen activator (T-plasminogen activator PLAT; TPA) |
| 118 | X99063 | Zyxin(ZYX) |
| | X14194 | Nidogen (NID); entactin (ENT) |
| 119 | Y15001 | Iroquois-related homeobox protein 3 (IRX3) |
| | Z23066 | Microphthalmia-associated transcription factor (MITF; MI); microphthalmia-related protein |
| | X73360 | Transducin-like enhancer of split protein 3 (TLE3; ESG) |

(continued on next page)

Table 2 (continued)

| Cluster No. | GenBank ID | Gene description |
|-------------|------------|---|
| | U65091 | Melanocyte-specific gene 1 (MSG1); Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (CITED1) |
| | X97986 | Desmocollin 1A/1B (DSC1) |
| | U81317 | Myelin-associated oligodendrocytic basic protein |
| | U21050 | TNF receptor-associated factor 3 (TRAF3); TRAFAMN; C040 receptor-associated factor 1 (CRAF1) |
| | D83966 | Protein tyrosine phosphatase |
| | L19622 | Tissue inhibitor of metalloproteinase 3 (TIMP3); SUN |
| | AF021031 | DiGeorge syndrome chromosome region 6 protein (DGCR6) |

plasticity of BMC. In the *F2* time period, clusters 13, 14, 15, 16, 17, 32, 33, and 51 were dramatically changed. In cluster 13, hepatocyte growth factor (HGF) was discovered. HGF is involved in hepatocyte proliferation [27,28]. HGF might also have an important role in GFP/CCl₄ model. In the *F3* time period, clusters 1, 2, 18, and 137 were focused. NumbL is involved in asymmetric division of nerve precursor cells [29]. The HOXD3 genes encode information important for determining the positional relationships of the antero-posterior axis in embryogenesis [30]. NumbL and HOXD3 might have an important role in regulating the plasticity of BMC. In *F4*, clusters 118, 119, 133, 134, 135, 136, 148, 149, 150, 151, 152, and 153 were found. In this period, genes involved in hepatocyte differentiation and homeostasis, such as GPI and HNF4, were focused [31]. This enzyme is essential for the glycolytic metabolism of hepatocytes. In the GFP/CCl₄ model, the level of albumin in bone marrow-derived hepatocytes increased significantly [12]. The fact that an enzyme such as GPI was induced at this period suggests that, at 4 weeks after BMC transplantation, transplanted BMCs begin to possess some of the metabolic functions of hepatocytes. HNF4- α was also upregulated in *F4* period. HNF4 plays an important role in the metabolic regulation of hepatocytes [32,33]. These results might be related with BMC differentiated into functional hepatocyte at this period in GFP/CCl₄ model. VEGF was also upregulated. VEGF promotes vasculogenesis and liver regeneration [34]. VEGF might also have an important role in accelerating the liver regeneration in GFP/CCl₄ model. Gene involved in inflammation such as TNF-R1 was also pointed out in GFP/CCl₄ model. TNF- α related inflammation signal is important in the generation of hepatoblast [17]. These results also showed that TNF- α related signal might be important for plasticity of BMC in GFP/CCl₄ model.

Here, we analyzed the change of molecular signature after BMC transplantation in GFP/CCl₄ model in mRNA level. Still many precise things are unconfirmed, but we think the information is useful to understand the mechanism of the plasticity of BMC in GFP/CCl₄ model. In the future, we are planning to further analyze the mechanisms.

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肝細胞癌に対する新しいバルーンマイクロカテーテルを用いた
リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法

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| 山崎 隆弘 | 木村 輝昭 | 浦田 洋平 | 丸本 芳雄 | 青山 浩司 | 石川 剛 |
| 田島 邦彦 | 横山雄一郎 | 大森 薫 | 川口浩太郎 | 高見 太郎 | 土屋 昌子 |
| 山口 裕樹 | 寺井 崇二 | 黒川 典枝 | 坂井田 功 | 沖田 極 | |

<短 報>

肝細胞癌に対する新しいバルーンマイクロカテーテルを用いた
リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法

山崎 隆弘¹⁾ 木村 輝昭²⁾ 浦田 洋平¹⁾ 丸本 芳雄¹⁾ 青山 浩司¹⁾ 石川 剛¹⁾
 田島 邦彦¹⁾ 横山雄一郎¹⁾ 大森 薫¹⁾ 川口浩太郎¹⁾ 高見 太郎¹⁾ 土屋 昌子¹⁾
 山口 裕樹¹⁾ 寺井 崇二¹⁾ 黒川 典枝¹⁾ 坂井田 功¹⁾ 沖田 極¹⁾

緒言：肝細胞癌に対して経皮的ラジオ波凝固療法(以下 RFA)が導入されて、約5年が経過している。その間にも腫瘍の凝固範囲を拡大させる工夫がさまざま報告されており¹⁻³⁾、われわれも肝動脈を遮断することで血流によるラジオエーター効果を低下させ、凝固範囲を拡大させる肝動脈バルーン閉塞下ラジオ波凝固療法(balloon-occluded RFA; BoRFA)を開発し、有用性について報告した^{4,5)}。しかし、血管歩行等により、すべての症例で固有肝動脈に通常のバルーンカテーテルを留置するのは不可能であり、そのような症例に対してわれわれはバルーンマイクロカテーテルを用いた肝動脈バルーン閉塞下ラジオ波凝固療法(balloon-microcatheter-occluded RFA; BMoRFA)を開発した⁶⁾。その際用いたバルーンマイクロカテーテルは、Commodore™ temporary occlusion balloon catheter (Cordis, Johnson and Johnson Co., USA)であったが、5 Fr カテーテルへの挿入が不可能であり、5.1 Fr カテーテルへの交換が必要で手技が煩雑な点、およびその後の検討で予想以上の凝固範囲拡大が得られなかった点⁷⁾が、問題点であり、改良の余地が必要であった。今回、上記の問題点を解決すべく、新しいバルーンマイクロカテーテルを用い、同療法にリピオドールを併用したりリピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法を考案したので報告する。

対象と方法：各種画像診断にて肝細胞癌と診断した4例(4結節)を対象とした。3例が女性、平均年齢69歳、腫瘍径31.3±11.9 mm(24-49 mm)、全例 Child-Pugh A であった。使用装置は、Radionics 社の Cool-tip RF System で、全例 3.0 cm 針を使用し、1回穿刺で12分間を基本凝固時間としたが、1例のみ腫瘍径が大きいため、引き抜いて12分間追加凝固を行った。使用したバルーンマイクロカテーテルは、Equinox™ occlusion balloon catheter (Micro Therapeutics, Inc. USA)の4×15 mm のバルーン径を用いた。今回の治療としては、既報⁴⁻⁶⁾と異なりリピオドール併用で行った。方法としては、通常の腹部血管造影の手技にて、5 Fr カテーテル(セレコンカテーテル：クリニカルサプライ)を用い、精査を行い、マイクロカテーテルにて Transcatheter arterial chemoembolization (TACE: eqirubicin hydrochloride, Mitomycin C, lipiodol 使用)を施行し、腫瘍内に lipiodol を pooling させた後、Equinox™ occlusion balloon catheter を担癌領域の肝動脈に留置し、バルーン閉塞後に RFA を施行した。同バルーンマイクロカテーテルは5 Fr カテーテルへの挿入が容易であり、バルーンは造影剤入り生理食塩水を0.2 cc 注入することで、

4×15 mm のバルーン径が得られた。治療効果判定は、1週間後の造影 CT にて施行し、凝固範囲を検討した。

結果：表1のごとく、12分間1回での凝固範囲が検討できたのは、症例2(図1)と4であり、それぞれ、50×47 mm、55×44 mm であった(症例3は、肝梗塞合併のため、計測していない)。同治療法1週間後の造影 CT では、全例で治療部位の早期濃染像は見られなかったが、症例4では、脈管近傍の safety margin が不足していたため、経皮的エタノール注入療法(PEI)を追加した。合併症では、症例1で発熱の遷延、症例3に肝梗塞を認めたが、保存的に改善した。その他は全例に発熱および疼痛を認めたが、対処療法で改善している。また、一時的に肝機能悪化は認めたが、約1週間後にはほぼ前値に復している。

考察：今回の RFA の工夫としては、新しいバルーンマイクロカテーテルを用いた点およびリピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法を考案した点の2つである。使用した新しいバルーンマイクロカテーテルは、以前使用した Commodore™ temporary occlusion balloon catheter とは違い、5 Fr カテーテルでの挿入が可能であり、手技の煩雑さが緩和された。今回の治療法は、肝動脈バルーン閉塞下ラジオ波凝固療法の変法として、ラジオエーター効果をより低下させる目的で、リピオドールを併用した。リピオドールは腫瘍内だけでなく、門脈枝にも流入し、腫瘍周囲の門脈血流を低下させること⁸⁾から、動脈および門脈血流の同時遮断効果⁹⁾を狙い、同療法を考案した。通常のバルーンカテーテル(バルーン径9 mm)での検討では、リピオドール併用肝動脈バルーン閉塞下ラジオ波凝固療法において非併用群と比較して、長径では有意差はないものの、短径で有意差を認めている(データ未発表)。リピオドール併用ラジオ波凝固療法での成績は、われわれは持ち合わせていないが、以前の報告¹⁰⁾よりも凝固範囲は広く、肝動脈閉塞を併用する意義は十分あるものと考ええる。以前の Commodore を用いた BMoRFA の1回凝固(12分間)の成績では長径38.3±5.7 mm、短径31.5±4.6 mm と、通常の RFA での凝固範囲(長径35.3±4.7 mm 短径25.9±3.7 mm)と比較して、短径のみの有意差であった⁷⁾。今回は1回凝固範囲の成績が2例のみであり、なおかつリピオドール併用であるが、50×47 mm、55×44 mm と十分な凝固範囲を認めている。また以前のバルーン径は4×10 mm であったが、新しいバルーンマイクロカテーテルは4×15 mm と短径は4 mm のままであるが、長径が15 mm と長くなっていることが、血流遮断に寄与し、凝固範囲の拡大につながったのかもしれない。今後症例を重ねた検討が必要であるが、新しいバルーンマイクロカテーテルは、通常の BoRFA 不可能例の適応に応用可能と考える。また、リピオドール併用肝動脈バルーン閉塞下ラ

¹⁾山口大学消化器病態内科学、²⁾同 漢方医学
 <受付日 2004年4月13日>

表1 症例の内訳と成績

| 症例 | 性別 | 年齢 | 腫瘍径 (mm) | 部位 | 凝固時間 (分) | TACE ^{a)} の量 | 凝固径 直径×短径×高さ (mm) |
|----|----|----|----------|----|----------|--|----------------------|
| 1 | 女性 | 70 | 49 | S4 | 24 | EPI ^{b)} 30 mg, MMC ^{c)} 6 mg, lipiodol 6.5 ml | 70×53×50 |
| 2 | 男性 | 67 | 27 | S5 | 12 | EPI ^{b)} 20 mg, MMC ^{c)} 4 mg, lipiodol 3 ml | 50×47×55 |
| 3 | 女性 | 69 | 25 | S8 | 12 | EPI ^{b)} 20 mg, MMC ^{c)} 4 mg, lipiodol 2 ml | — |
| 4 | 女性 | 70 | 24 | S5 | 12 | EPI ^{b)} 20 mg, MMC ^{c)} 4 mg, lipiodol 2.5 ml | 55×44×55 |

a) TACE : transcatheter arterial chemoembolization, b) EPI : epirubicin hydrochloride, c) MMC : mitomycin C

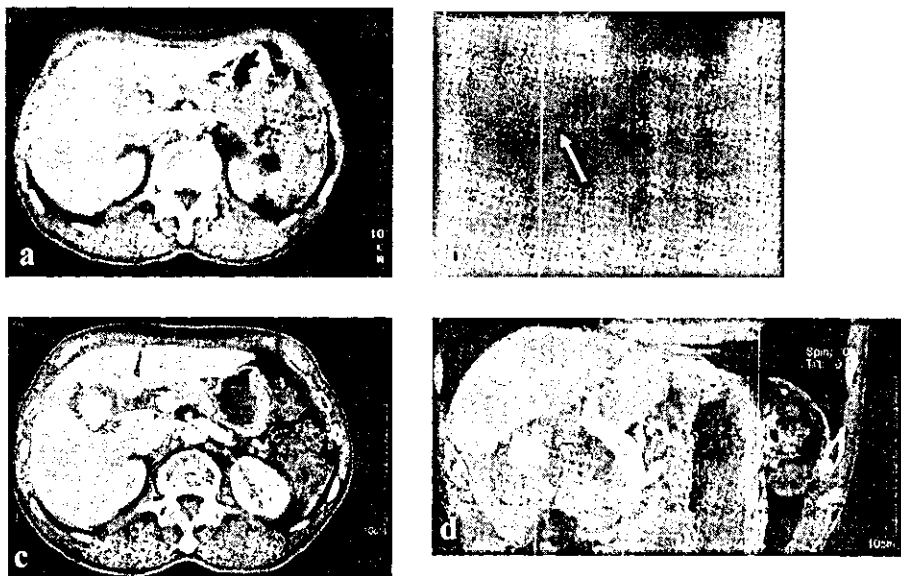



図1 症例2 ; a : 治療前CTA, b : 右肝動脈にバルーンマイクロカテーテルを挿入した所 (矢印), c(axial像), d(sagittal像) : 治療後のdynamic CT 50×47×55 mmの凝固範囲を認める

ジオ波凝固療法は、バルーンマイクロカテーテルでの手技でも有効性が示唆された。なお、同治療法は、血管造影検査を行う侵襲性および高価なバルーンマイクロカテーテルを用いる経済面ではデメリットではあるが、最近報告されている5 cm展開針RITA Model 90の単独治療の1回凝固と比較し、凝固範囲が同等以上である点¹¹⁾および短期間での精査と治療が可能となる点では十分なメリットがあり、今後はとくに大型肝癌に適応拡大可能と考える。

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TEL(03)3664-7900(代表)

【禁 無断転載・複製】

自己骨髄細胞を用いた肝硬変症に対する肝臓再生療法

寺井崇二 大森 薫 石川 剛
 青山浩司 高見太郎 横山雄一郎
 田島邦彦 坂井田功 沖田 極

肝不全患者に対し生体肝移植が行われているが、ドナーの問題、外科侵襲の多さという問題点があり、次世代の肝臓再生療法の開発が求められている。われわれは骨髄細胞（肝幹細胞）の移植療法の有効性を評価するため、GFP/CCl₄モデルを開発し解析してきた。さらに基盤研究を基に臨床研究（自己骨髄細胞を用いた肝臓再生療法）へと展開した。われわれは、肝移植が受けられない肝不全患者に対する新たな治療方法の開発を慎重に進めている。

はじめに

骨髄中の幹細胞の存在が報告されて以来、再生医療の新たな細胞源として骨髄細胞が注目されている。われわれは『自己骨髄細胞を用いた肝臓再生療法』の臨床開発を進めるため、5年前より基礎研究を行い、実際に肝硬変症に対する骨髄移植の有効性の検討を骨髄細胞からの肝細胞への分化増殖の *in vivo* 評価モデルを開発し検討してきた^{1,2)}。このモデルにおいては、持続的な四塩化炭素 (CCl₄) 投与による肝障害時に骨髄細胞から肝細胞への分化・増殖が確認され、骨髄細胞投与後、肝臓内に投与された骨髄細胞は肝細胞索構造を構築した。発生段階の肝芽細胞の増殖に SEK1, MKK7→SAPK/JNK→c-Jun シグナル系が肝芽細胞の自己複製と生存維持に必須の役割を果たしていること³⁾、持続炎症時に骨髄細胞のアルブミン陽性肝細胞への分化が確認できたことは相似点があると考えられた。さらにわれわれのモデルでは、骨髄細胞投与により、血清アルブミン値の回復や生存率も骨髄細胞の非投与群に比べ有意に改善していた。また肝線維化も有意に改善していた⁴⁾。これらの結果から、自己骨髄細胞を用いた肝臓再生療法は臨床応用可能な次世代の移植医療に

なりうると考えられた。一方で過去にすでに、循環器領域の虚血性疾患に対しても自己骨髄細胞の注入療法が行われており^{5,6)}、また骨髄移植そのものについてはすでに20年以上の実績がある。われわれは基礎研究の結果を基盤とし平成13(2001)年12月に、『自己骨髄細胞を用いた肝臓再生療法』の臨床研究について、山口大学医学部生命倫理委員会に申請し、安全性・有用性の臨床研究の認可を受けた。さらに約2年の期間をかけ、実際に臨床研究を行うための細胞療法部関連の設備、器具を整え、臨床研究の準備を行い、平成15年11月14日に69歳男性に対して国内最初の「自己骨髄細胞を用いた肝臓再生療法」の phase I 研究を開始した。本稿ではこの第1症例の経過について提示する。

臨床研究の対象症例

今回の臨床研究における対象患者（非代償性肝硬変症）は以下のとおりとした。

T. B. は 3 mg/dL 以下、血小板は 5 万以上、肝細胞癌についてはコントロールできている症例。食道、胃静脈瘤のコントロールが良好であり、また骨髄採取に伴い全身麻酔をかけるので心肺機能は良好の患者とした。

てらいしゅうじ、おおもり かおる、いしかわ たけし、あおやま こうじ、たかみ たろう、よこやま ゆういちろう、たじま くにひこ、さかいだいさお、おきた きわむ：山口大学医学部先端分子応用医科学講座（消化器病態内科学）

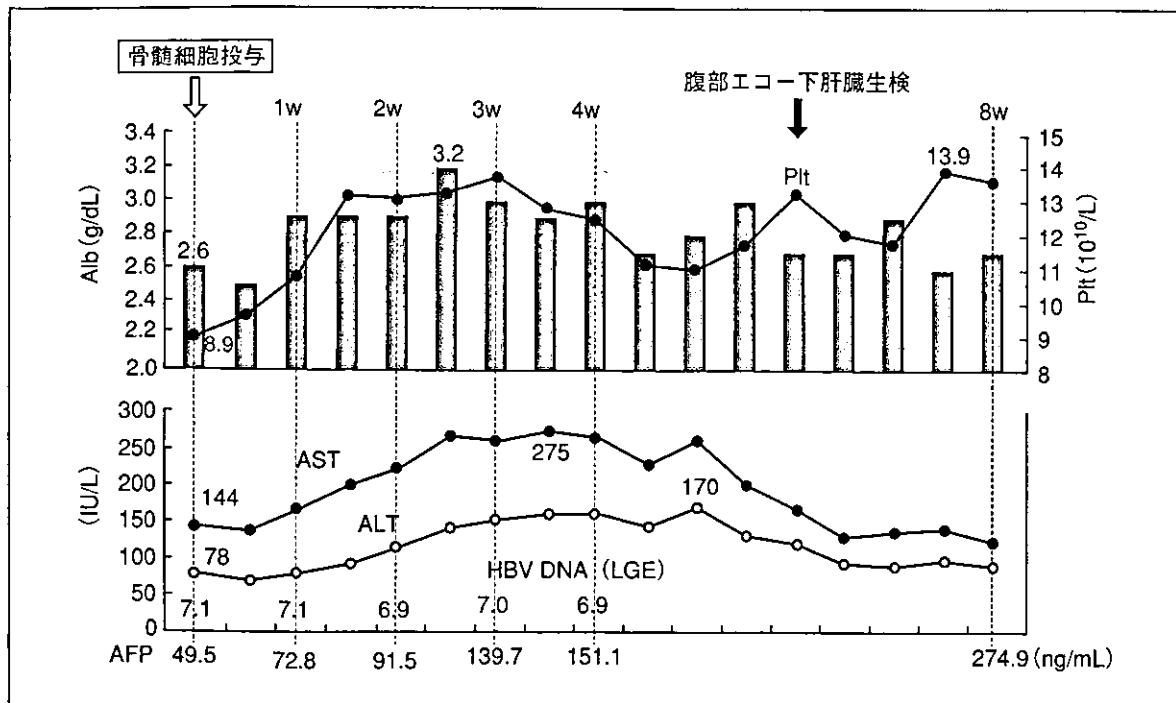


図 1 症例の臨床経過

症例：臨床経過

HBV 陽性非代償性肝硬変症の 69 歳男性に対し、全身麻酔下にて自己骨髄細胞を 400 mL 採取、洗浄後有核細胞の 4×10^9 個を採取した。採取した自己骨髄細胞を約 1 時間かけ末梢静脈から投与し、肝臓の再生の誘導の有無について評価した。術中特に大きな合併症もなく、術後血液検査にて骨髄細胞の肝再生に与える影響について評価した。その結果、基礎研究の結果より予測されたように、骨髄細胞投与後より血清アルブミン値は、術前 2.5 g/dL であったものが最大 3.2 g/dL まで改善、また腹部エコーにて腹水の減少を確認し利尿薬の投与量の減量に成功した。また図 1 に経過を示すように AFP (α -フェトプロテイン) も骨髄細胞投与により増加した(図 1)。その他、血小板の値も改善した。また骨髄細胞投与後に施行した肝生検において、hepatocyte nuclear factor-4 (HNF4), AFP などの誘導も確認され、肝再生が誘導された可能性が考えられた。

考 察

症例については術後の経過もよく、骨髄細胞の投与により肝臓再生が誘導された可能性が示唆され

た。現時点においては、われわれの臨床研究は安全性評価の phase I 研究であり、現在までの 6 例の検討では重篤な副作用の発生はない。今後は慎重に症例を重ね phase I 臨床研究を進めていき、肝臓領域における新たな再生療法としての『自己骨髄細胞を用いた肝臓再生療法』の開発の可能性を評価していきたい。

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