fibrillary acidic protein (GFAP), Musashi, Nestin, PDGFRα and CD133, and have the ability to differentiate into neurons and oligodendrocytes (13,14). GFAP is also a marker of astrocytes, suggesting that the neural stem cells are somewhat related to astrocytes (15). The cells are isolated from the subventricular zone or the hippocampus in the brain. It was recently reported that Sox2-positive neural stem cells in the adult hippocampus are multipotent and can self-renew, whereas Sox2 deficiency causes neurodegeneration and impaired neurogenesis, indicating the physiological significance of Sox2 for the stemness of neural stem cells (14,16). Therefore, Sox2 may be useful as a marker of neural stem cells.

D. Embryonic stem cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of a blastocyst, an early stage embryo. Wide varieties of embryonic stem cell lines have been characterized (17,18), and the some of the key genes for maintaining the undifferentiated state and pluripotency have been described as *POU5F1 (OCT4)*, *NANOG*, *SOX2*, and others such as *ZFP42 (REX1)*, *UTF1*, *GDF3*, *FOXD3*, *TRET*, *FGF4* (19,20,21,22). The assessment of several human embryonic stem cell lines has established SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, POU5F1 (OCT4) and NANOG as common markers for human embryonic stem cells (23). It is also suggested that GJA1 is a marker for the undifferentiated state of human embryonic stem cells (24). A report on gene expression in human embryonic stem cells and human embryonic carcinoma cells showed that POU5F1 (OCT4) is upregulated in both types of cells, as compared to control samples including both somatic cell lines and normal testis (25).

E. Induced pluripotent stem cells

Induced pluripotent stem cells are another type of pluripotent stem cells, which are artificially reprogrammed from non-pluripotent cells and resemble human embryonic stem cells in phenotypic features. The retroviral introduction of *Pou5f1 (Oct3/4)*, *Sox2*, *Myc (c-myc)* and *Klf4* was reported to develop mouse induced pluripotent stem cells (26). The expression of a set of factors, namely *POU5F1 (OCT4)*, *SOX2*, *KLF4* and *MYC (c-Myc)* or the set of *POU5F1 (OCT4)*, *SOX2*, *NANOG* and *LIN28* was shown to induce reprogramming of human fibroblasts to pluripotent stem cells by retroviral transduction (27,28). More recently, valproic acid, a histone deacetylase inhibitor, was found to enable reprogramming of primary human fibroblasts with only two factors, *Oct4* and *Sox2*, without the need for the oncogenes *c-Myc* or *Klf4* (29). Reprogramming of liver and stomach cells (30) and generation of mouse-induced pluripotent stem cells without viral vectors (31) or retroviral integration (32) have also been reported.

3. PHYSIOLOGICAL ASPECTS OF STEM CELLS

A. Proliferation of stem cells

It is known that, with increasing passages of mesenchymal stem cells in culture, the proliferation rate and the capacity for differentiation decrease (33). These changes are associated with expression of several genes. For example, the expression of nephroblastoma overexpressed gene and EPH receptor A5 in human bone marrow mesenchymal stem cells is increased in late stage of cultures, whereas the expression of runt-related transcription factor 2 and necdin homolog (mouse) is decreased (34). Genome change in the cells also occurs in some cases; however, it is not well known whether this phenomenon is universal.

Mouse hematopoietic stem cells are known to proliferate in relatively slow cell cycle kinetics compared to multipotent progenitors *in vivo* (35). The gene expression pattern of hematopoietic stem cells also differs in the proliferating state *in vivo* (36). An analysis of *Foxo3a*$^{-/-}$ mice showed that Foxo3a is important in maintaining the self-renewal capacity of hematopoietic stem cells, although the proliferation of the cells was not affected by Foxo3a deletion (37). Human embryonic stem cells can be usually cultured more than 30 to 50 passages (22). It has been shown that human embryonic stem cells require feeder cells to grow and are negative for SSEA-1. Although mouse embryonic stem cell growth is also feeder-cell dependent, mouse cells do express SSEA-1 (38). LIF (leukemia inhibitory factor) is known to be an important factor for maintaining the self-renewal capacity of mouse embryonic stem cells. The morphology of mouse embryonic stem cells is relatively diverse, whereas human embryonic stem cells are round with sharp boundaries. The expression of SSEA-4 and vimentin is specific for human embryonic stem cells (39). It has also been reported that a retinoblastoma protein is important for the proliferation of monolayer cultures of embryonic stem cell-derived cardiomyocytes (40).

B. Differentiation of stem cells

The features of stem cells that distinguish them in different species include direction for differentiation and gene expression. In osteogenic differentiation of mesenchymal stem cells, the expression of ID4, CRYAB and SORT1 are altered (41). Embryonic stem cells have the capacity for multilineage differentiation, such as ectoderm, mesoderm and endoderm. The differentiation is induced by transfer of the cells from fibroblast feeder layers, which maintain stemness of embryonic stem cells, to suspension culture (42,43). It was reported that neuronal differentiation is induced with FGF-2 and medium conditioned by HepG2 (44). Furthermore, induced pluripotent stem cells generated from patients with amyotrophic lateral sclerosis are reported to be differentiated into motor neurons (45). Gene expression in human embryonic stem cells is altered during differentiation. NODAL, LEFTY A, LEFTY B and PITX2 are described as marker genes for the differentiation of embryoid bodies, which are multicellular aggregates of differentiated and undifferentiated cells (46). Genes such as Hex or Hnf6/Oc-1 play an important role

during the differentiation of liver and pancreas from their progenitors (47).

4. CANCER STEM CELLS

A. Factors distinguishing cancer stem cells from normal stem cells

Recent research implicates the involvement of cancer stem cells in cancer. Cancer stem cells share features with normal stem cells. The differences in their features, however, are under investigation. Even though the origin of cancer stem cells is not well understood, several suggestions related to their microenvironment (niche) have been proposed: [1] niche around normal stem cells allows cancer stem cells to grow, [2] cancer stem cells arise from normal stem cells that adopt an alternative niche and [3] niche-independent cancer stem cells arise from normal stem cells or [4] cancer stem cells arise from progenitor cells (48). It has been shown that embryonic stem cell-like gene sets including Sox2, c-Myc, Dnmt1, Cbx3, Hdac1 and Yy1 are activated in human epithelial cancers, and c-Myc increases the fraction of tumour-initiating cells in primary human keratinocytes transformed by Ras and IκBα (49).

B. Cancer stem cells in cancer

The population of cancer stem cells in cancer is very rare. Cancer stem cells are defined as cells with stem cell features that have the capacity of tumourigenesis in immunodeficient mice (50,51). Research on human embryonal carcinoma cells, which are the stem cells of teratocarcinomas, has shown that these cells express SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, similar to human embryonic stem cells (38,52,53). To identify cancer stem cells from solid tumours, cells are sorted with surface markers. CD133 and CD44, which are markers for stem cells, are often used as surface markers to identify cancer stem cells from tumours. In one report, the $CD133^+$ subpopulation from human brain tumours was shown to be tumourigenic, whereas the $CD133^-$ subpopulation did not have tumour-initiation capability (54). Cancer stem cells are also known to exist in the side population fraction (55,56,57). In addition, breast cancer cells with the $CD44^+CD24^{-/low}$ phenotype have a higher tumourigenic capacity as compared to other populations of cancer cells, and the gene sets expressed in the $CD44^+CD24^{-/low}$ population are related to metastasis-free survival and overall survival (58).

5. CONCLUSION

In conclusion, stem cells, which have the capacity for self-renewal and differentiation, show various profiles in gene expression. Each kind of stem cell has unique aspects, but they also share common features. Recent research advances have added to our knowledge of the role of cancer stem cells in cancer_based on the concept of cancer stem cell niche.

REFERENCES

1. Weissman, I. L. *Cell*, **100**, 157-168 (2000)
2. Pittenger, M. F., Mackay, A. M., Beck,

S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. Marshak, D. R. *Science*, **284**, 143-147 (1999)
3. Ringdén, O., Uzunel, M., Rasmusson, I., Remberger, M., Sundberg, B., Lönnies, H., Marschall, H.-U., Dlugosz, A., Szakos, A., Hassan, Z., Omazic, B., Aschan, J., Barkholt, L., Le Blanc, K. *Transplantation*, **81**, 1390-1397 (2006)
4. Kumar, S., Chanda, D., Ponnazhagan, S. *Gene Ther.*, **15**, 711-715 (2008)
5. Tsai, M., Hwang, S., Chen, K., Lee, Y., Hsu, L., Chang, Y., Wang, C., Peng, H., Chang, Y., Chao, A., Chang, S., Lee, K., Wang, T., Wang, H., Soong, Y. *Stem Cells*, **25**, 2511-2523 (2007)
6. Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., Verfaillie, C. M. *Nature*, **418**, 41-49 (2002)
7. Young, H. E., Steele, T. A., Bray, R. A., Hudson, J., Floyd, J. A., Hawkins, K., Thomas, K., Austin, T., Edwards, C., Cuzzourt, J., Duenzl, M., Lucas, P. A., Black, A. C. *Anat. Rec.*, **264**, 51-62 (2001)
8. Bryder, D., Rossi, D.J., Weissman, I.L. *Am. J. Pathol.*, **169**, 338-346 (2006)
9. Majeti, R., Park, C. Y., Weissman, I. L. *Cell Stem Cell*, **1**, 635-645 (2007)
10. Baum, C. M., Weissman, I. L., Tsukamoto, A. S., Buckle, A. M., Peault, B. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2804-2808 (1992)
11. Walker, M. R., Patel, K. K., Stappenbeck, T. S. *J. Pathol.*, **217**, 169-180 (2009)
12. Laurenti, E., Vamum-Finney, B., Wilson, A., Ferrero, I., Blanco-Bose, W. E., Ehninger, A., Knoepfler, P. S., Cheng, P., MacDonald, H. R., Eisenman, R. N., Bernstein, I. D., Trumpp, A. *Cell Stem Cell*, **3**, 611-624 (2008)
13. Yadirgi, G., Marino, S. *J. Pathol.*, **217**, 242-253 (2009)
14. Suh, H., Consiglio, A., Ray, J., Sawai, T., D'Amour, K. A., Gage, F. H. *Cell Stem Cell*, **1**, 515-528 (2007)
15. Gage, F. H. *Science*, **287**, 1433-1438 (2000)
16. Ferri, A. L. M., Cavallaro, M., Braida, D., Cristofano, A. D., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P. P., Sala, M., DeBiasi, S., Nicolis, S. K. *Development*, **131**, 3805-3819 (2004)
17. The International Stem Cell Initiative *Nat. Biotech.*, **25**, 803-816 (2007)
18. The Steering Committee of the International Stem Cell Initiative *Nat. Biotech.*, **23**, 795-797 (2005)
19. Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., Smith, A. *Cell*, **95**, 379-391 (1998)
20. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., Smith, A. *Cell*, **113**, 643-655 (2003)
21. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., Yamanaka, S. *Cell*, **113**, 631-642 (2003)
22. Brimble, S. N., Zeng, X., Weiler, D. A., Luo, Y., Liu, Y., Lyons, I. G., Freed, W. J., Robins, A. J., Rao, M. S., Schulz, T. C. *Stem*

Cells Dev., **13**, 585-596 (2004)
23. Allegrucci, C., Young, L. E. *Hum. Reprod. Update*, **13**, 103-120 (2007)
24. Bhattacharya, B., Miura, T., Brandenberger, R., Mejido, J., Luo, Y., Yang, A. X., Joshi, B. H., Ginis, I., Thies, R. S., Amit, M., Lyons, I., Condie, B. G., Itskovitz-Eldor, J., Rao, M. S., Puri, R. K. *Blood*, **103**, 2956-2964 (2004)
25. Sperger, J. M., Chen, X., Draper, J. S., Antosiewicz, J. E., Chon, C. H., Jones, S. B., Brooks, J. D., Andrews, P. W., Brown, P. O., Thomson, J. A. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 13350-13355 (2003)
26. Takahashi, K., Yamanaka, S. *Cell*, **126**, 663-676 (2006)
27. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. *Cell*, **131**, 861-872 (2007)
28. Park, I., Zhao, R., West, J. A., Yabuuchi, A., Huo, H., Ince, T. A., Lerou, P. H., Lensch, M. W., Daley, G. Q. *Nature*, **451**, 141-147 (2008)
29. Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., Melton, D. A. *Nat. Biotech.*, **26**, 1269-1275 (2008)
30. Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T., Yamanaka, S. *Science*, **321**, 699-702 (2008)
31. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., Yamanaka, S. *Science*, **322**, 949-953 (2008)
32. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., Hochedlinger, K. *Science*, **322**, 945-949 (2008)
33. Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A., Nikbin, B. *BMC Cell Biol.*, **7**, 14 (2006)
34. Tanabe, S., Sato, Y., Suzuki, T., Suzuki, K., Nagao, T., Yamaguchi, T. *J. Biochem.*, **144**, 399-408 (2008)
35. Cheshier, S. H., Morrison, S. J., Liao, X., Weissman, I. L. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 3120-3125 (1999)
36. Nygren, J. M., Bryder, D., *PLoS ONE*, **3**, e3710 (2008)
37. Miyamoto, K., Araki, K. Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., Chen, C., Hosokawa, K., Nakauchi, H., Nakayama, K., Nakayama, K. I., Harada, M., Motoyama, N., Suda, T., Hirao, A. *Cell Stem Cell*, **1**, 101-112 (2007)
38. Pera, M. F., Reubinoff, B., Trounson, A. *J. Cell Sci.*, **113**, 5-10 (2000)
39. Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerecht-Nir, S., Amit, M., Hoke, A., Carpenter, M. K., Itskovitz-Eldor, J., Rao, M. S. *Dev. Biol.*, **269**, 360-380 (2004)
40. Yamanaka, S., Zahanich, I., Wersto, R. P., Boheler, K. R. *PLoS ONE*, **3**, e3896 (2008)
41. Kulterer, B., Friedl, G., Jandrositz, A., Sanchez-Cabo, F., Prokesch, A., Paar, C., Scheideler, M., Windhager, R., Preisegger, K.-H., Trajanoski, Z. *BMC Genomics*, **8**, 70 (2007)
42. Odorico, J. S., Kaufman, D. S., Thomson, J. A. *Stem Cells*, **19**, 193-204 (2001)
43. Trounson, A. *Endocr. Rev.*, **27**, 208-219 (2006)
44. Schulz, T. C., Palmarini, G. M., Noggle, S. A., Weiler, D. A., Mitalipova, M. M.,

Condie, B. G. *BMC Neurosci.*, **4**, 27 (2003)
45. Dimos, J. T., Rodolfa, K. T., Niakan, K. K., Weisenthal, L. M., Mitsumoto, H., Chung, W., Croft, G. F., Saphier, G., Leibel, R., Goland, R., Wichterle, H., Henderson, C. E., Eggan, K. *Science*, **321**, 1218-1221 (2008)
46. Dvash, T., Mayshar, Y., Darr, H., McElhaney, M., Barker, D., Yanuka, O., Kotkow, K. J., Rubin, L. L., Benvenisty, N., Eiges, R. *Hum. Reprod.*, **19**, 2875-2883 (2004)
47. Zaret, K. S., Grompe, M. *Science*, **322**, 1490-1494 (2008)
48. Clarke, M. F., Fuller, M. *Cell*, **124**, 1111-1115 (2006)
49. Wong, D. J., Liu, H., Ridky, T. W., Cassarino, D., Segal, E., Chang, H. Y. *Cell Stem Cell*, **2**, 333-344 (2008)
50. Eaves, C. J. *Nature*, **456**, 581-582 (2008)
51. Quintana, E., Shackleton, M., Sabel, M. S., Fullen, D. R., Johnson, T. M., Morrison, S. J. *Nature*, **456**, 593-599 (2008)
52. Andrews, P. W. *Phil. Trans. R. Soc. Lond. B*, **357**, 405-417 (2002)
53. Andrews, P. W., Matin, M. M., Bahrami, A. R., Damjanov, I., Gokhale, P., Draper, J. S. *Biochem. Soc. Trans.*, **33**, 1526-1530 (2005)
54. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., Dirks, P. B. *Nature*, **432**, 396-401 (2004)
55. Miki J., Rhim J. S. *Prostate Cancer Prostatic Dis.*, **11**, 32-39 (2008)
56. Harris, M. A., Yang, H., Low, B. E., Mukherje, J., Guha, A., Bronson, R. T., Shultz, L. D., Israel, M. A., Yun, K. *Cancer Res.*, **68**, 10051-10059 (2008)
57. Wu C., Alman, B. A. *Cancer Lett.*, **268**, 1-9 (2008)
58. Liu, R., Wang, X., Chen, G. Y., Dalerba, P., Gurney, A., Hoey, T., Sherlock, G., Lewicki, J., Shedden, K., Clarke, M. F. *N. Engl. J. Med.*, **356**, 217-226 (2007)

—Reviews—

医薬品のウイルス安全性確保：核酸増幅検査（NAT）によるC型肝炎ウイルス検出の評価とNATによる高感度検出のためのウイルス濃縮法の開発

内田恵理子,*,[a] 山口照英[b]

Viral Safety of Biologicals: Evaluation of Hepatitis C Virus (HCV) Nucleic Acid Amplification Test (NAT) Assay and Development of Concentration Method of HCV for Sensitive Detection by NAT

Eriko UCHIDA*,[a] and Teruhide YAMAGUCHI[b]

[a]*Division of Cellular and Gene Therapy Products, and* [b]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

(Received August 28, 2009)

The most important issue for the safety of biological products and blood products derived from human sources is how to prevent transmission of infectious agents. The hepatitis C virus (HCV) is a major public health problem due to its high prevalence. HCV is mainly transmitted by exposure to blood and highly infectious during the early window period with extremely low viral loads. Therefore it is important to develop more sensitive detection methods for HCV. In the case of blood products, both serological test and nucleic acid amplification test (NAT) are required to detect HCV. Since NAT is highly sensitive, establishment of a new standard is required for validation of NAT assay. NAT guideline and establishment of the standard for HCV RNA and HCV genotype panel is introduced in this review. On the other hand, to enhance the sensitivity of virus detection by NAT, a novel viral concentration method using polyethyleneimine (PEI)-conjugated magnetic beads (PEI beads) was developed. PEI beads concentration method is applicable to a wide range of viruses including HCV. Studies using the national standard for HCV RNA, HCV genotype panel and seroconversion panel, suggest that virus concentration method using PEI-beads is useful for improvement of the sensitivity of HCV detection by NAT and applicable to donor screening for HCV.

Key words——hepatitis C virus (HCV); viral safety; nucleic acid amplification test (NAT); standard; polyethyleneimine (PEI); virus concentration

1. はじめに

ヒト由来成分を原料とする医薬品の安全性確保における最重要課題はウイルス等の感染症の伝播をいかに防止するかである．C型肝炎ウイルス（HCV）はわが国では約200万人が感染していると推定されている感染頻度の極めて高いウイルスで，主として血液を介して感染する．輸血によるHCV感染リスクは，1990年初頭に導入された血清学的検査に加えて1999年に核酸増幅検査（NAT）が導入されたことによって極めて低減化された．しかしながら，感染初期のウインドウ期のHCVは極めて低濃度のウイルス量で感染が成立し，チンパンジーを用いた感染実験では50 mlの血漿中に含まれるわずか1-5コピーのHCVウイルスゲノムで感染が認められるとの報告もあり，[1] ウインドウ期によるHCV感染を防ぐには，より高感度・高精度なウイルス検出手法の開発が求められている．PCRを始めとするNAT法は数コピーから数十コピーという微量のウイルスゲノムを検出できる高感度検出法であり，「生物由来原料基準」（平成15年厚生労働省告示第210号2003年5月20日制定，平成17年厚生労働省告示第177号2005年3月31日改正）及び「血漿分画製剤のウイルスに対する安全性確保に関するガイドラインについて」（平成11年8月30日医薬発第1047号厚生省医薬安全局長通知）により，血液製剤（輸血用血液製剤，血漿分画製剤）では血清学的検査に加えてNATによるHCV検査が義務付け

[a]国立医薬品食品衛生研究所遺伝子細胞医薬部，[b]同生物薬品部（〒158-8501 東京都世田谷区上用賀1-18-1）
*e-mail: uchida@nihs.go.jp

本総説は，日本薬学会第129年会シンポジウムS33で発表したものを中心に記述したものである．

られている．一方で，NATは極めて高感度であるため，その分析法の評価にはこれまでにない基準が必要とされる．

本総説では，医薬品のウイルス安全性確保という観点から，NATによるHCV検出を評価するためのNATガイドラインとNATの評価に必要となる標準品やパネル血漿の作製について紹介するとともに，NATの高感度化のためのウイルス濃縮法の開発とHCVの高感度検出への応用に関する筆者らの研究について紹介する．

2. ヒト由来成分を原料とする医薬品の安全性確保とNATによるHCV検出

2-1. 医薬品のウイルス安全性確保に関する基準・指針 ヒト由来成分を原料とする医薬品のウイルス安全性確保に関連する基準や指針としては，前述の「生物由来原料基準」や「ヒト（同種）由来細胞や組織を加工した医薬品または医療機器の品質及び安全性の確保について」（平成20年9月12日薬食発第0912006号厚生労働省医薬食品局長通知）などが策定されている．これらの基準や指針により，ヒト由来成分を原料とするヒト尿由来製品，ヒト（同種）由来細胞組織加工医薬品等においてもHCVはB型肝炎ウイルス（HBV），ヒト免疫不全ウイルス（HIV）等と同様，NATによる検査が義務化あるいは強く推奨されている（Table 1）．

2-2. NATの検出感度とウインドウ期 NATとはNucleic acid Amplification Test（核酸増幅検査）のことで，ウイルスなどの微量の遺伝子（核酸）を人工的に増幅して高感度に検出する方法の総称である．DNAポリメラーゼを用いたサーマルサイクル反応によりDNAを増幅するPCR法（Polymerase Chain Reaction：ポリメラーゼ連鎖反応）が一般によく知られているが，ほかにも恒温で核酸を増幅するTMA法（Transcription-mediated Amplification：転写媒介増幅法），LAMP法（Loop-mediated Isothermal Amplification：鎖置換反応法），ICAN法（Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid：等温遺伝子増幅法），NASBA法（Nucleic Acid Sequence-Based Amplification：核酸配列増幅法）やDNAリガーゼを用いたサーマルサイクル反応により核酸を増幅するLCR法（Ligase Chain Reaction：リガーゼ連鎖反応法）などの様々な方法が開発され，ウイルス検査に利用されている．各増幅法の原理や特徴については他著に譲る．[2]

NATはウイルス核酸を増幅して検出するため，抗体を検出する血清学的検査に比べて極めて高感度であり，ウインドウ期を短縮することが可能である．HCVの場合，抗体検査のウインドウ期は約82日とされるが，日本赤十字社（日赤）ではNATの実施によりウインドウ期が約25日と大幅に短縮された[3]（ただし，これは検査法に依存した数値でありすべてに当てはまるわけではない）．日赤におけるHCVのNATによる検出感度は74 IU/mlとされる．[4] 一方，「四課長通知」（血漿分画製剤のウイルス安全対策について：平成15年11月7日薬食審査発第1107001号，薬食安発第1107001号，薬食監発第1107001号，薬食血発第1107001号，厚生労働省医薬食品局審査管理課長，安全対策課長，監視指導・麻薬対策課長，血液対策課長通知）によると，HCVのNATによる検出感度としてはHBV，HIVとともに100 IU/mlが求められている．昨年，日赤では新しい検査試薬を導入するとともに，検査に用いる検体の容量を現在の4倍以上に増やすことでNATの検出感度は一段と向上したという．さらに今後，ウイルス濃縮法を含む様々な手法により検出感度を上げることができれば，ウインドウ期のさらなる短縮が可能と考えられる．

2-3: NATガイドライン 上述の通り，血液製剤やヒト尿由来製品では，数コピーから数十コピーという微量のウイルス遺伝子の検出が要求されるNATがスクリーニング検査として義務付けられているが，この場合，検出感度等の適切な精度管理

Table 1. Requirements for Virus Test in Biological Products

- Blood Products for Transfusion
  —NAT for HBV-DNA, HCV-RNA, and HIV-RNA with individual or mixed blood
- Plasma Derived Medicines
  —NAT for HBV-DNA, HCV-RNA and HIV-RNA with original plasma
- Human Urine Derived Products
  —NAT for HBV-DNA, HCV-RNA, HIV-RNA with pooled urine at appropriate timing
- Cell or Tissue Derived Products
  —Interview or screening for HBV, HCV, HIV, HTLV and parvovirus B19

が極めて重要である．そこで，血液製剤の安全性確保を目的としてNATを行う場合に適切な精度管理が実施されるよう，検査精度の確保及び試験方法の標準化のための方策を示したものが「血液製剤のウイルスに対する安全性確保を目的とした核酸増幅検査（NAT）の実施に関するガイドライン」（平成16年8月3日薬食発第0803002号厚生労働省医薬食品局長通知）である．本ガイドラインは「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン」を補完するものであり，血液製剤のドナースクリーニング検査，原料血漿の製造工程への受入れ時の試験，血漿分画製剤の製造過程における工程内管理試験や最終製品の検査としてNATを行う場合に適用される．HCV, HBV, HIV及びその他の準用可能なウイルスが対象となる．

ウイルス遺伝子の検出を目的とする定性検出法としてのNATの検証で重要な項目は，①特異性，②検出感度，③頑健性である．NATにおける特異性とは，試料中に共存すると考えられる物質の存在下で目的とするウイルス遺伝子のみを確実に検出できる能力であり，類似のウイルスに対する交差反応性（非特異的反応）がないこと，目的とするウイルスの種々の遺伝子型を検出できることを適当な参照パネル（ジェノタイプパネル）を用いて証明することが求められる．

検出感度とは，試料中に含まれる目的ウイルス遺伝子の検出可能な最低の量のことを指し，NATでは95％の確率で検出される検体一定量当たりのウイルス遺伝子の最低量である陽性カットオフ値を検出感度として設定する．検出感度は一般に標準品の希釈系列を作製して求める必要がある．ランコントロールには，95％の確率で検出される検出感度の3倍量のウイルスを含む標準検体を用いることが推奨されている．

頑健性とは，分析条件の小さな変動が結果に影響しないという信頼性を表すものであり，陰性試料及び陽性試料（95％の確率で検出される検出感度の3倍量のウイルスをスパイクしたもの）を，それぞれ少なくとも20検体を用いて試験を実施し，すべての陰性試料が陰性となり，すべての陽性試料が陽性となることによって示すことができる．

NATガイドラインでは，検査精度の確保及び試験方法の標準化のための方策として，上記の要件のほかに，核酸の抽出・増幅及び増幅産物の検出の最適化，従事者の技術の標準化，汚染防止のための施設・設備の整備等に関する要件等も示されている．

2-4. NAT試験用標準品，参照品について

NATガイドラインに従ってNATの検出感度や精度を比較・評価するには，基準となる標準品あるいは標準物質（参照品）が必要となる．標準品としては，①国際標準品，②国際標準品とのデータの互換性が保証された国内標準品，③国際標準品又は国内標準品とのデータの互換性が保証された自社標準物質（参照品）等のいずれかを使用することが求められ，WHO（World Health Organization）や国内においてNAT試験用のウイルス標準品の作製が行われている（Table 2）．WHOの国際標準品はNIBSC（National Institute for Biological Standards and Control）によりHCV, HBV, HIV, HAV及びパルボウイルスB19について作製されている．国内標準品は厚生労働省薬事分科会血液事業部会安全技術調査会小委員会によりHCV, HBV及びHIVについて策定されている．HCV RNAの国際標準品としては，1997年にジェノタイプ1aを用いて第一次国際標準品（96/790）が樹立され，現在は第三次国際標準品（00/560）がNIBSCより入手可能である．一方，HCV RNAの第一次国内標準品（JCV-1b No122）は国内に多いジェノタイプ1bを

Table 2. International and National Standards for Virus DNA/RNA NAT Assays

	WHO International Standard (NIBSC)	National Standard
HCV	06/100 Genotype 1a 154881 IU/ml	JCV-1b No122 Genotype 1b 100000 IU/ml
HBV	97/746 Genotype A, HBsAg subtype adw 5×10^5 IU/vial	HBV-129 Genotype C, HBsAg subtype adr 4.4×10^5 IU/ml
HIV	97/650 HIV-1, Genotype B 5.56 $\log_{10}$ IU/vial	HIV-00047 HIV-1, Genotype B 1.4×10^5 IU/ml
HAV	00/560 5×10^4 IU/vial	—
Parvovirus B19	99/800 5×10^5 IU/vial	—

用いて1999年に作製された．HCV RNA国内標準品の作製には，日常的にHCV-NATを実施している国内外の7施設が参加し，各施設が任意の核酸抽出・増幅法を用いて第一次WHO国際標準品（96/790）を基準に国内標準品候補品の力価を算出することにより，その平均値から国内標準品の力価が100 000 IU/mlと決定された．[5] 現在，HCV RNA国内標準品は感染症研究所から入手可能である．

一方，NATの特異性の評価，ジェノタイプ毎の検出感度の評価に用いる参照パネルの作製状況をTable 3に示す．国際参照パネルはHCVとHIVについて用意されている．国内参照パネルはHCV, HIV, HBVの標準パネル血漿がいずれも厚生労働科学研究費補助金「安全な血液製剤を確保するための技術の標準化及び血液製剤の精度管理法の開発に関する研究」（主任研究者吉澤浩司）[6]により作製されており，今後公開予定とされる．HCV国内標準パネル血漿の詳細をTable 4に示す．この標準パネル血漿は献血された新鮮凍結血漿をもとに作製されたもので，HCV抗体が出現する前のウインドウ期の血漿とキャリア期の血漿に，感染既往期の血漿及び陰性対照血漿を加えた計100本が選定されている．HCVパネルには国内に存在する代表的なジェノタイプである1a, 1b, 2a, 2bの4種類が網羅されている．標準パネル血漿のHCV RNA量（copies/ml）は，報告書[6]にある換算表によりWHOの力価（IU/ml）との相互換算が可能である．

2-5. 血液製剤等のHCV安全対策　血液製剤等のウイルス安全対策として，製造メーカーには，①国内標準品や適当な参照パネルを用いて，各社で採用しているNATのバリデーションを実施し，当該NATの検出限界が100 IU/mlの精度となるよう精度管理を行うこと，②血漿分画製剤の製造工程には，ウイルスが十分に除去・不活化されていることを確認できる，少なくとも10^9以上のウイルスクリアランスを示す製造工程を導入することが前述の「四課長通知」により求められている．また，輸血用血液製剤については，医療機関は患者に対して輸血前後のHCV, HBV, HIVの検査を実施することが「血液製剤等に係る遡及調査ガイドライン」（平成17年3月10日薬食発第0310012号厚生労働省医薬食品局長通知，平成20年12月26日一部改正）により求められている．

一方，国は血漿分画製剤メーカーや輸血前後のウイルス検査を実施する機関に対して，NATの品質管理に係るコントロールサーベイを実施している．コントロールサーベイとは，HCV, HBV, HIVそれぞれの国内標準品を血漿で希釈した検体を参加各施設にブラインドで配布して試験を実施することにより，各施設で実施しているNATの感度・精度等の状況を把握し，必要な対策を取るための調査試験の性格を持ち，これによりNATの検出感度の向上及び標準化に努めている．

Table 3. International and National Reference Panels

	International Reference Panel (NIBSC)	National Reference Panel[6]
HCV	02/202 6 samples (6 major genotypes) non WHO reference material	100 samples (5 genotypes and negative controls)
HIV	01/466 11 samples (10 different genotypes and a negative control) WHO international standard	100 samples (subtype A, B, E, negative control)
HBV	—	100 samples (genotype A, B, C, D, F; subtype adw, adr, adr mutant, ayr, negative)

Table 4. HCV RNA National Genotype Panel for Standardization of NAT Assay

Genotype	Classification*	Panel Number	HCV RNA (copies/ml)
1a	carrier period	2	8.1×10^5～1.0×10^6
1b	window period	12	9.0×10^2～6.9×10^7
	carrier period	5	4.5×10^3～2.9×10^7
1b+2a	window period	1	4.5×10^7
2a	window period	11	1.9×10^5～6.7×10^7
	carrier period	10	3.2×10^5～5.2×10^7
2b	window period	8	4.8×10^5～8.5×10^7
	carrier period	3	3.2×10^6～2.3×10^7
—	anamnestic infection	46	Not detected
	negative control	2	Not detected

* window period: HCVAb < 1.0; carrier, anamnestic infection: HCVAb ≧ 1.0. Data were collected from the original report.[6]

3. NATによるHCV検出の高感度化のためのウイルス濃縮法の開発

NATは目的とする遺伝子を数コピーから数十コピーという高感度で検出できる方法であるが，検出限界よりさらに低い濃度のウイルスが存在する場合には検出不可能である．最初に述べたように，感染初期のウインドウ期のHCVは極めて低濃度のウイルス量でも感染が成立すること，また細胞組織加工医薬品のような製品ではウイルスの不活化・除去が行えないことから，可能な限り高感度なウイルス否定試験の開発が望まれている．

NATによるウイルス検出をより高感度化する方法の1つの方法として，ウイルスを濃縮後に検出することで検査にかける検体の用量を増加させる方法がある．われわれは新規ウイルス濃縮法として，ポリエチレンイミン結合磁気ビーズ(PEI磁気ビーズ)を用いた手法を開発し，HCVを始め多くのウイルスがPEI磁気ビーズに吸着して濃縮可能であり，NATによるウイルス検出を高感度化できることを報告した（Table 5）.[7,8] ウイルス濃縮法の原理としては，主としてPEIの陽性荷電とウイルス表面分子の陰性荷電との静電気的相互作用によりウイルスがPEIに吸着して濃縮されると考えている(Fig. 1).

HCVの濃縮については，細胞組織加工医薬品の試験への適用を想定した培養上清中のHCV，及びドナースクリーニングへの適用を想定した血漿中のHCVのいずれの場合も，PEI磁気ビーズによりほぼ定量的に濃縮されることが確認された（Fig. 2）．HCVの検出感度をHCV RNA国内標準品を用いて検討した結果，1 mlのウイルス液からPEI磁気ビーズで10倍濃縮を行うことにより検出感度が向上し，1 IU/mlがほぼ確実に検出可能となった．ま

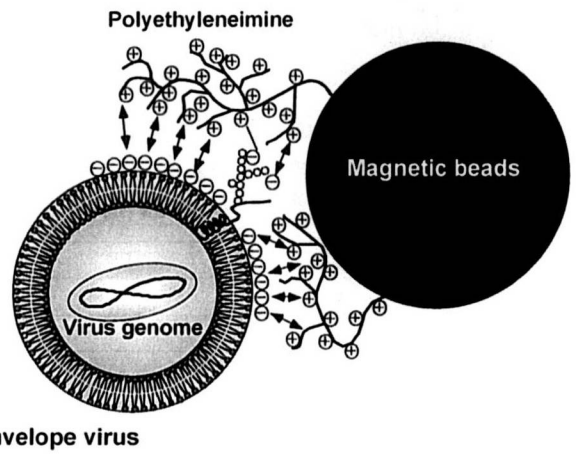

Fig. 1. Mechanism of Virus Concentration by PEI Beads

Table 5. Summary of Concentration of Viruses by PEI beads

Viruses	Natural host	Virus genome	Envelope	Size (nm)	PEI-beads concentration
Model Viruses					
Cytomegalovirus (CMV)	Simian	DNA	+	180–200	+
Herpes Simplex Virus Type-I (HSV-1)	Human	DNA	+	150–200	+
Vesicular Stomatitis Virus (VSV)	Bovine	RNA	+	70–150	+
Amphotropic Murine Leukemia Virus	Murine	RNA	+	80–110	+
Sindbis Virus	Human	RNA	+	60–70	+
Adenovirus Type 5	Human	DNA	−	70–90	+
Simian Virus 40 (SV40)	Simian	DNA	−	40–50	+
Porcine Parvovirus (PPV)	Porcine	DNA	−	18–24	+
Poliovirus Sabin 1	Human	RNA	−	25–30	+*
Human Hepatitis Viruses					
Hepatitis B Virus (HBV)	Human	DNA	+	40–45	+*
Hepatitis C Virus (HCV)	Human	RNA	+	40–50	+
Hepatitis A Virus (HAV)	Human	RNA	−	25–30	+

* Concentrated by the addition of antibodies.

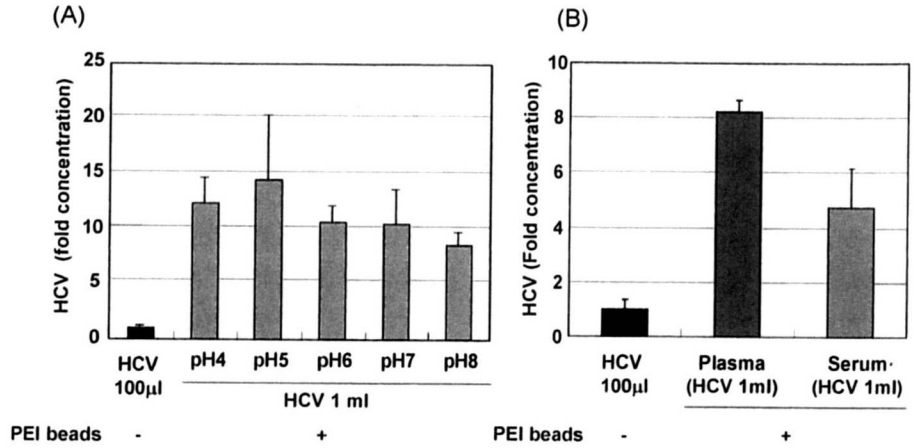

Fig. 2. HCV concentration by PEI beads
(A) HCV was spiked in cell culture medium containing 2% fetal bovine serum. (B) HCV was spiked in human plasma or human serum.

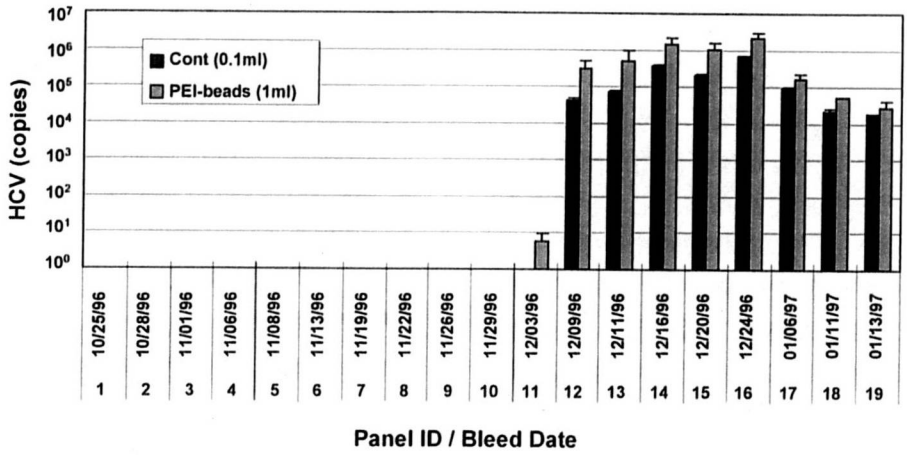

Fig. 3. Application of PEI Beads Concentration Method to HCV Seroconversion Panel

た，HCV 濃縮の特異性を検討するため，市販の HCV ジェノタイプパネルからジェノタイプや由来する国の異なる 10 種類のパネル血漿を選んで 10 倍濃縮を行ったところ，すべて 5 倍以上の濃縮が得られ，ジェノタイプが異なるものでも適用可能であることが示された．さらに，HCV のセロコンバージョンパネル（HCV 感染後のウインドウ期の初期に短期集中して採血されたシングルドナー血漿）を用いて検出の有効性を検討した結果，PEI 磁気ビーズ濃縮を行うことにより，濃縮せずに直接検出した時と比べて 6 日早く採血された検体についても HCV が検出可能となり，ウインドウ期が短縮された (Fig. 3)．これらの結果から，PEI 磁気ビーズ濃縮法は NAT による HCV 検出の高感度化に有用であり，医薬品のウイルス安全性確保に重要なドナーのスクリーニングにも適用可能と考えられる．

4. おわりに

血液製剤等のウイルス安全性確保を目的として HCV の検査に NAT が導入されたことにより，HCV の検出は高感度化されウインドウ期は短縮された．しかしなお NAT には検出限界があり，ウインドウ期をなくすことはできないため安全性確保はいまだ十分とは言えない．より一層の安全性を確保するには，現在よりさらに高感度・高精度なウイルス検出手法の開発が望まれる．NAT によるウイルス検出技術やその周辺技術は急速に進展しており，ウイルス濃縮法を含め様々な手法の開発が進められている．最新の技術を取り入れ，技術の進歩に即応した医薬品のウイルス安全対策が進められることが望まれる．

謝辞　PEI 磁気ビーズによるウイルス濃縮法の

開発は，日本赤十字中央研究所の佐藤巧栄博士，岩田明子氏及びJSR㈱の日方幹雄博士，村田充宏博士との共同研究の成果であり，この場を借りて感謝申し上げます．本研究の一部は，厚生労働科学研究費及びヒューマンサイエンス振興財団政策創薬総合研究事業の支援を受けて行われたものです．

REFERENCES

1) Busch M. P., Hirschkorn D. F., Herring B. L., Delwart E. L., McAuley J., Murthy K. K., Alter H. J., *Transfusion*, **43** (9s), 32A (2003).
2) Hayakawa T., Yamaguchi T., Oshizawa T., Ishii-Watabe A., *Iyakuhin Kenkyu*, **33**, 275–284, (2002).
3) ⟨http://www.tokyo.bc.jrc.or.jp/tmpfile/yougo/yousetsu_aa-oo.htm#uu-3⟩, cited 04 December, 2009.
4) Yokoyama S., *Jpn. J. Transfusion Med.*, **48**, 279–285 (2002).
5) Mizusawa S., Okada Y., Horiuchi Y., Tanaka T., Sato K., Kaneko K., Sasaki Y., Tanaka T., Tomono T., Tomomizu T., Hayami S., Hijikata M., Hirako I., Mayumi M., Mikami K., Mishiro S., Miyamoto S., Muta K., Weimer T., Gierman T., Komuro K., Yamaguchi T., *Jpn. J. Transfusion Med.*, **51**, 515–519 (2005).
6) Yoshizawa K.: ⟨http://mhlw-grants.niph.go.jp/index.html⟩, MHLW grants system database No. 200301268A., cited 04 December, 2009.
7) Satoh K., Iwata A., Murata M., Hikata M., Hayakawa T., Yamaguchi T., *J. Virol. Methods*, **114**, 11–19 (2003).
8) Uchida E., Kogi M., Oshizawa T., Furuta B., Satoh K., Iwata A., Murata M., Hikata M., Yamaguchi T., *J. Virol. Methods*, **143**, 95–103 (2007).

Mutation Research 673 (2009) 9–20

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Dose-dependent alterations in gene expression in mouse liver induced by diethylnitrosamine and ethylnitrosourea and determined by quantitative real-time PCR☆

Takashi Watanabe[a], Gotaro Tanaka[b], Shuichi Hamada[c], Chiaki Namiki[d], Takayoshi Suzuki[e], Madoka Nakajima[f], Chie Furihata[a,*]

[a] Functional Genomics Laboratory, School of Science and Engineering, Aoyama Gakuin University, Fuchinobe 5-10-1, Sagamihara, Kanagawa 229-0006, Japan
[b] Tokushima Research Center, Taiho Pharmaceutical Co. Ltd., Hiraishiebisuno 224-2, Kawauchichou, Tokushima, Tokushima 771-0194, Japan
[c] Genetic Toxicology Group, Toxicology Division II, Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd., Sunayama 14, Kamisu-shi, Ibaraki 314-0255, Japan
[d] Central Research Laboratory, SSP Co. Ltd., Nanpeidai 1143, Narita, Chiba 286-8511, Japan
[e] Division of Cellular & Gene Therapy Products, National Institute of Health Sciences, Kamiyoga 1-18-1 Setagaya-ku, Tokyo 158-8501, Japan
[f] Genetic Toxicology Group, Biosafety Research Center, Foods, Drugs, and Pesticides, Shioshinden 582-2, Fukude-cho, Iwata-gun, Shizuoka 437-1213, Japan

ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 31 October 2008
Accepted 9 November 2008
Available online 21 November 2008

Keywords:
Genotoxic carcinogens
Dose-dependent alteration
Hierarchical clustering
k-means clustering
IPA

ABSTRACT

We examined the dose-dependency of gene expression changes for 51 genes in mouse liver treated with two *N*-nitroso genotoxic hepatocarcinogens, diethylnitrosamine (DEN) and ethylnitrosourea (ENU) by quantitative real-time PCR (qPCR). DEN (3, 9, 27 and 80 mg/kg bw) or ENU (6, 17, 50 and 150 mg/kg bw) was injected intraperitoneally into groups of five male 9-week-old B6C3F$_1$ mice and the livers were dissected after 4 h and 28 days. Total RNA from pooled livers was reverse-transcribed to cDNA and the amount of each gene was quantified by qPCR. Results were analyzed by hierarchical and *k*-means clustering and ingenuity pathway analysis (IPA). The most characteristic result was a similar dose-dependency of gene expression changes with DEN and ENU. Twenty-one genes exhibited a distinct dose-dependent increase in expression at 4 h for both carcinogens [*Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Cyp4a10*, *Cyp21a1*, *Fos*, *Gadd45b*, *Gdf15*, *Hmox1*, *Hspb1*, *Isg20l1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Ppp1r3c*, *Rcan1* and *Tubb2c*], although the increase in gene expression due to ENU was generally weaker than that due to DEN. Only *Gdf15* showed a dose-dependent increase in expression at 28 days for both carcinogens. The differences between DEN and ENU were in the expression of additional genes (7 for DEN and 8 for ENU). IPA extracted five gene networks: Network-1 included genes related to cancer and cell cycle arrest and associated with *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gadd45b*, *Gdf15*, *Hspb1*, *Mdm2* and *Plk2* and Network-2 was related to DNA replication, recombination, repair and cell death and associated with *Cyp21a1*, *Gdf15*, *Ppp1r3c*, *Rcan1* and *Tubb2c*. The present results show a distinct dose-dependency of gene expression changes induced by DEN and ENU. These changes were associated with cancer, cell cycle arrest, DNA replication, recombination, repair and cell death and were seen not only at 4 h but also, for some, at 28 days after administration.

1. Introduction

Diethylnitrosamine (DEN) and ethylnitrosourea (ENU) are potent genotoxic *N*-nitroso carcinogens that induce hepatocellular carcinomas in mouse liver [1,2]. It has been reported that after its metabolic biotransformation, DEN produces the promutagenic adducts O^6-ethylguanine (O^6-EtG) and O^4- and O^2-ethylthymine and that O^4-ethylthymine may be responsible for the initiation of hepatocellular carcinomas in rats [3]. ENU, which is a direct-ethylating agent, forms several major adducts upon reaction with DNA, of which O^6-EtG, O^4- and O^2-ethylthymine and N^3-ethylthymine have been implicated in mutagenic lesions [4]. Suzuki et al. have reported that mutagenic activity by DEN and ENU was clearly detected with the *lacZ* mutation assay in mouse liver at 7 days [5]. Mientjes et al. have reported that the O^6-EtG levels increased as early as 1.5 h after treatment, whereas at 3 days more than 90% of the lesions had been removed from the DNA in the livers of DEN- and ENU-treated mice, based on *lacZ* transgenic mice [6]. After this period, however, with the bulk of O^6-EtG removed, the induction of *lacZ* mutations was observed at 3 days and continued to increase for some weeks.

☆ This work was a JEMS/MMS/Toxicogenomics group collaborative study.

* Corresponding author at: Department of Chemistry and Biological Science, School of Science and Engineering, Aoyama Gakuin University, 5-10-1 Fuchinobe, Sagamihara, Kanagawa 229-8558, Japan. Tel.: +42 759 6233; fax: +42 759 6511.
E-mail address: chiefurihata@gmail.com (C. Furihata).

1383-5718/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2008.11.004

Previously, Waring et al. showed by DNA microarray that a number of genes are up-regulated and down-regulated in rat liver, with rats dosed daily with DEN for 3 days and euthanized on the 4th day [7]. Genes up-regulated by DEN included genes related to growth arrest and DNA damage, such as *Bax*, *Ccnd1*, *Ccng1*, *Cdkn1a/p21*, *Gadd45* and *Jun*. However, no studies have focused on either the DNA damaging time of 4 h or the mutation fixing time of 28 days in DEN-treated mouse or rat liver. Although it has been reported that ENU induced expression of *Bax*, *Crp*, *Cyp2a*, *Gstm2*, *Icam1*, *Mig*, and *Mt2* mRNA in mouse liver, little is known about differential gene expression in ENU-exposed rodent liver [8].

Quantitative real-time PCR (qPCR) is an alternative technology for toxicogenomics [9]. qPCR is a highly regarded and reliable quantitative method but analysis of a large number of genes may be lengthy. It is impractical to examine a great number of genes with qPCR. Therefore, we selected 51 candidate genes (Table 1) based on our previous results using the Affymetrix GeneChip Mu74AV2 and original DNA microarray to determined the effects of DEN, dimethylnitrosamine, dipropylnitrosamine, ENU, *o*-aminoazotoluene, 7,12-dimethylbenz[*a*]anthracene, dibenzo[*a*,*l*]pyrene, phenobarbital and ethanol exposure in mouse liver for 4 and 20 h and 14 and 28 days in our JEMS/MMS/Toxicogenomics group collaborative study; results were reported in part [10]. We examined gene expression changes at an early time after administration, as we were interested in whether toxicogenomics was useful for carcinogen screening. In the previous study, using a single dose for each chemical, gene expression changes in number and degree were observed to peak at 4 h after administration. It is known that genotoxic *N*-nitroso carcinogens induce DNA damage and repair in a matter of a few hours after their administration; DNA adducts [6], DNA strand-breaks [11], unscheduled DNA synthesis [12] and other lesions have been reported. It is also known that mutations are observed in transgenic mouse liver 28 days after genotoxic *N*-nitroso carcinogen administration [5,6]. However, related gene expression changes at these time points have not yet been fully elucidated.

Table 1
Fifty-one genes examined in the present study.

No.	Symbol	Gene name	Accession number
1	*Bax*	Bcl2-associated X protein	NM_007527
2	*Bcl2*	B-cell leukemia/lymphoma 2	NM_009741
3	*Btg2*	B-cell translocation gene 2, anti-proliferative	NM_007570
4	*Casp1*	IL-1B converting enzyme; interleukin 1 beta-converting enzyme	NM_009807
5	*Ccnf*	Cyclin F	NM_007634
6	*Ccng1*	Cyclin G1	NM_009831
7	*Ccng2*	Cyclin G2	NM_007635
8	*Cdkn1a (p21)*	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669
9	*Cyp1a1*	Cytochrome P450, family 1, subfamily a, polypeptide 1	NM_009992
10	*Cyp1a2*	Cytochrome P450, family 1, subfamily a, polypeptide 2	NM_009993
11	*Cyp4a10*	Cytochrome P450, family 4, subfamily a, polypeptide 10	NM_010011
12	*Cyp21a1*	Cytochrome P450, family 21, subfamily a, polypeptide 1	NM_009995
13	*Dpyd*	Dihydropyrimidine dehydrogenase	NM_170778
14	*Egfr*	Epidermal growth factor receptor	NM_207655
15	*Ephx1*	Epoxide hydrolase 1, microsomal	NM_010145
16	*Fabp5*	Fatty acid binding protein 5, epidermal	NM_010634
17	*Fos*	FBJ osteosarcoma oncogene	NM_010234
18	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	NM_008655
19	*Gadd45g*	Growth arrest and DNA-damage-inducible 45 gamma	NM_011817
20	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084
21	*Gdf15*	Growth differentiation factor 15	NM_011819
22	*Glul*	Glutamate-ammonia ligase (glutamine synthetase)	NM_008131
23	*Gstk1*	Glutathione S-transferase kappa 1	NM_029555
24	*Gyk*	Glycerol kinase	NM_212444
25	*Hist1h1c*	H1 histone family, member 2	NM_015786
26	*Hspa1b (Hsp70)*	Heat shock protein 1B	NM_010478
27	*Hspb1*	Heat shock protein 1	NM_013560
28	*Hspb2 (Hsp27)*	Heat shock protein 2	NM_024441
29	*Hmox1*	Heme oxygenase (decycling) 1	NM_010442
30	*Hprt1*	Hypoxanthine guanine phosphoribosyl transferase 1	NM_013556
31	*Igfbp1*	Insulin-like growth factor binding protein 1	NM_008341
32	*Isg20l1*	Interferon stimulated exonuclease gene 20-like 1	NM_026531
33	*Jun*	Jun oncogene	NM_010591
34	*Kras*	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	NM_021284
35	*Lig3*	Ligase III, DNA, ATP-dependent	NM_010716
36	*Lrp1*	Low density lipoprotein receptor-related protein 1	NM_008512
37	*Mbd1*	Methyl-CpG binding domain protein 1	NM_013594
38	*Mdm2*	Transformed mouse 3T3 cell double minute 2	NM_010786
39	*Myc*	Myelocytomatosis oncogene	NM_010849
40	*Net1*	Neuroepithelial cell transforming gene 1	NM_019671
41	*Pdgfb*	Platelet-derived growth factor, B polypeptide	NM_011057
42	*Plk2*	Polo-like kinase 2; serum-inducible kinase	NM_152804
43	*Pml*	Promyelocytic leukemia	NM_008884
44	*Pmm1*	Phosphomannomutase 1	NM_013872
45	*Ppp1r3c*	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	NM_016854
46	*Rad52*	RAD52 homolog (S. cerevisiae)	NM_011236
47	*Rcan1(Dscr1)*	Regulator of calcineurin 1	NM_019466
48	*Trp53*	Transformation related protein 53	NM_011640
49	*Tubb2c*	Tubulin, beta 2c	NM_146116
50	*Ube2e1 (UbcM3)*	Ubiquitin-conjugating enzyme E2E 1, UBC4/5 homolog (yeast)	NM_009455
51	*Ung*	Uracil-DNA glycosylase	NM_011677

In this paper, we report our studies of gene expression changes in B6C3F$_1$ mouse liver induced by multiple doses of two typical alkylating agents, DEN and ENU. We investigated the dose-dependency of gene expression changes at two different time points: 4 h, characterized by the production of many DNA lesions, and 28 days, characterized by fixing of mutations [6]. If we could show dose-dependency in gene expression changes at 4 h, we could clarify key genes related to DNA lesions and subsequent various phenomena in liver cells induced by DEN and ENU. If we could show the dose-dependency in gene expression changes at 28 days, we could clarify key genes related to effects of mutations and subsequent changes that may be causal for carcinogenesis. Our purpose is to determine biological cell responses induced by DEN and ENU by examining the dose-dependency at these two time points.

In addition, we examined gene networks using IPA to elucidate interactions between genes with altered expression.

2. Materials and methods

2.1. Animal treatment

Male B6C3F$_1$ mice were obtained at 8 weeks of age from Charles River Japan, Inc. (Yokohama, Japan). They were kept in plastic cages on wood chips as bedding and given food (Oriental MF, Oriental Yeast Co., Tokyo) and water *ad libitum* in an air-conditioned room [12 h light (7 a.m. to 7 p.m.), 12 h dark; 23 ± 2 °C; 55 ± 5% humidity]. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the Mitsubishi Chemical Safety Institute Ltd.

Mice at 9 weeks of age were injected intraperitoneally (i.p.) with DEN (3, 9, 27 and 80 mg/kg bw; Wako Pure Chem. Ind. Ltd., Osaka, Japan; CAS 55-18-5) dissolved in sterile water or ENU (6, 17, 50 and 150 mg/kg bw; Wako Pure Chem. Ind. Ltd., Osaka, Japan; CAS 759-73-9) dissolved in sterile water. Control animals for the DEN- and ENU-treated groups received sterile water. At 4 h and 28 days after treatment, animals were sacrificed after which the liver was collected, frozen on dry ice, and stored at −80 °C until use.

2.2. RNA isolation and relative quantification by real-time PCR

To isolate total RNA, approximately 150 mg from each liver (main lobe) was placed into TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and immediately homogenized using a Potter homogenizer. The samples were further homogenized with a 1 ml syringe and 18 gauge needle. Finally, total RNA was purified using an ethanol precipitation method. Complementary DNA (cDNA) was yielded from total RNA using the SuperScript First strand synthesis system for RT-PCR kit (Invitrogen Corp.).

qPCR amplifications were performed in triplicate using the SYBR Green I assay in an Opticon II (MJ Research, Inc., Waltham, MA, USA). The reactions were carried out in a 96-well plate in 20-μl reactions containing 2× SYBR Green Master Mix (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA), 2 pmol each of forward and reverse primer, and a cDNA template corresponding to 10 ng total RNA. Each primer sequence and Ct value are shown in Table 2. We selected 51 genes based on our previous results from the original DNA microarray and Affymetrix GeneChip Mu74AV2 for samples after treatment of DEN, dimethylnitrosamine, dipropylnitrosamine, ENU, *o*-aminoazotoluene, 7,12-dimethylbenz[*a*]anthracene, dibenzo[*a*,*l*]pyrene, phenobarbital and ethanol in our JEMS/MMS/Toxicogenomics group collaborative study. *Gapdh* and *Hprt1* were selected as housekeeping genes. SYBR Green PCR conditions were 95 °C for 10 min, followed by 95 °C for 10 s, 58 °C for 50 s and 72 °C for 20 s, for 45 cycles. In each assay a standard curve was determined concurrently with examined samples. In the preliminary experiment the highest group was selected for each gene and was used as the standard sample in the subsequent assay. In each standard curve determination, there were six dilution series of standard samples, diluted up to 1/5, 1/25, 1/125, 1/625 and 1/3125 of the selected standard liver cDNA for each gene. Finally, relative quantitative values of each sample were determined with 1/25 diluted cDNA and were normalized with those of the *Gapdh* genes. Relative *Gapdh* expression levels of experimental groups are presented in Fig. 1.

2.3. Data analysis and clustering algorithm

For the cluster analysis program, we performed a logarithmic ($\log_2$) transformation of the data to stabilize the variance and the gene expression profile of each DEN- and ENU-treated sample, normalized to the median gene expression level for the entire sample set. Both hierarchical and *k*-means clustering were performed using GENESIS software (http://genome.tugraz.at/) [13] for each data set at 4 h and 28 days separately. Gene groups were presented automatically by hierarchical clustering. Four clusters were set up initially in *k*-means clustering based on hierarchical clustering results. Genes which belonged to dose–response groups by both clustering methods were defined as dose–response genes. Furthermore, genes which showed less than a 0.5-fold decrease dose-dependently were evaluated as decrease genes by expression pattern because the decrease genes were few and could not be extracted using both clustering methods.

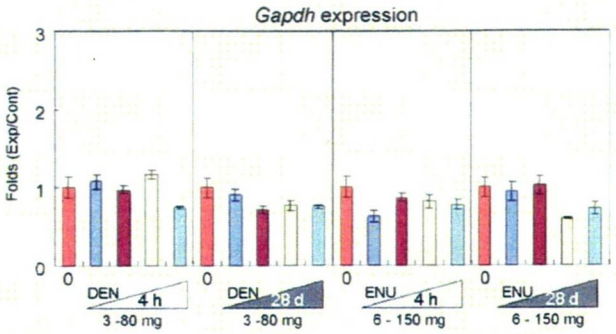

Fig. 1. Relative expression of *Gapdh*. DEN (0–80 mg/kg bw) and ENU (0–150 mg/kg bw) were given to 9-week-old mice (five per group). Total RNA was extracted from pooled liver and reverse-transcribed to cDNA. *Gapdh* expression was determined by qPCR in triplicate assays. Results are shown as mean ± S.D.

The color displays given in Fig. 2 show the $\log_2$ (expression ratio) as (1) red when the treatment sample is up-regulated relative to the control sample, (2) blue when the treatment sample is down-regulated relative to the control sample and (3) white when the $\log_2$ (expression ratio) is close to zero.

2.4. Pathway analysis

Numerical experimental data at 4 h and 28 days after DEN or ENU treatment were separately analyzed by ingenuity pathway analysis (IPA) Software-Complete Pathways Database. These data were generated through the use of IPA, a web-delivered application (www.Ingenuity.com) that enables the visualization and analysis of biologically relevant networks to discover, visualize, and explore therapeutically relevant networks. IPA information was extracted by experts from the full text of the scientific literature, including information about genes, drugs, chemicals, cellular and disease processes, and signaling and metabolic pathways.

Expression data sets containing gene identifiers (Entrez gene identifiers) and their corresponding expression values as fold changes were uploaded as a tab-delimited text file. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. To start building networks, the application program queries the Ingenuity Pathways Knowledge Base for interactions between focus genes and all other gene objects stored in the knowledge base and generates a set of networks. The program then computes a score for each network according to the fit of the network to the set of focus genes. The score indicates the likelihood of the focus genes in a given network being found together due to random chance. A score of >2 indicates that there is a <1 in 100 chance that the focus genes were assembled randomly into a network due to random chance.

3. Results

3.1. Dose-dependent alteration of gene expression induced by DEN

3.1.1. Clustering analysis for gene expression

Unsupervised hierarchical clustering results are shown in Fig. 2. The changes in gene expression are represented colorimetrically as described in Section 2. The clustering presented four groups (DEN-4 h-Grp-1 to DEN-4 h-Grp-4) and an ungrouped gene 4 h after administration, and three groups (DEN-28 d-Grp-1 to DEN-28 d-Grp-3) and eight ungrouped genes 28 days after administration. As unsupervised hierarchical clustering was performed for 4 h and 28-day samples separately, group member genes were different for 4 h groups and 28-day groups.

At 4 h, all 20 DEN-4 h-Grp-1 genes showed a dose-dependent increase of more than 3–64-fold. Twelve DEN-4 h-Grp-2 genes were suggested to have a gradual dose-dependent increase of less than that for the expression in DEN-4 h-Grp-1. Two DEN-4 h-Grp-4 genes exhibited a dose-dependent decrease of less than 0.3-fold.

Table 2
Primer sequences of 51 genes examined in the study.

No.	Symbol	Left	Right	Ct
1	*Bax*	CCAGGATGCGTCCACCAAGAAG	GGAGTCCGTGTCCACGTCAGC	28
2	*Bcl2*	GATGACTTCTCTCGTCGCTACC	CATCCCTGAAGAGTTCCTCCAC	31
3	*Btg2*	ACGGGAAGAGAACCGACATGC	ATGATCGGTCAGTGCGTCCTG	24
4	*Casp1*	GTCTTGGAGACATCCTGTCAGG	GCATCTGTAGCCTAAATTCTGG	32
5	*Ccnf*	AGCACAAAGCCTTGCCACCATC	AAGCCAGGTGCGTGTCCTTGTC	25
6	*Ccng1*	TGGCCGAGATTTGACCTTCTGG	GTGCTTCAGTTGCCGTGCAGTG	22
7	*Ccng2*	GCCATCAAGCTAGGACTGTTAG	CACTTATCAACTCCATTCCCTG	26
8	*Cdkn1a (p21)*	TCCCGTGGACAGTGAGCAGTTG	CGTCTCCGTGACGAAGTCAAAG	22
9	*Cyp1a1*	TGGCCGATCGGAGGTCTTTC	AAGTGTTCACAGCGGGCGTG	29
10	*Cyp1a2*	GATGCTCTTCGGCTTGGGAAAG	CCATAGTTGGGTGTCAGGTCCAC	20
11	*Cyp4a10*	AGCCACAAGGGCAGTGTTCAGG	CCAAGCGGCCATTGGAAGAAAG	23
12	*Cyp21a1*	TGTGCTGCCCTTAAAGAAGAGTG	TTGAGCATCCCGTTCCCGTTTC	25
13	*Dpyd*	GTGCGGCTAAAGGCTGATGTGG	CCCATGGTTCACTGGTTTGCATG	24
14	*Egfr*	AGAACGCCTTCCACAGCCAC	ACTCTCGGAACTTTGGGCGG	22
15	*Ephx1*	CATTGTCTCCTCCCAGCGCTTC	GGGCATGCAGGATCTCAGAAGG	21
16	*Fabp5*	ACGGTCTGCACCTTCCAAGACG	ACCCGAGTGCAGGTGGCATTG	24
17	*Fos*	GTCGACCTAGGGAGGACCTTAC	CATCTCTGGAAGAGGTGAGGAC	31
18	*Gadd45b*	TGTACGAGGCGGCCAAACTG	TGTCGCAGCAGAACGACTGG	28
19	*Gadd45g*	GGAAAGCACAGCCAGGATGCAG	ATTCAGGACTTTGGCGGACTCG	26
20	*Gapdh*	GCTCTCAATGACAACTTTGTCAAG	CTTCCTTGGAGGCCATGTAGGC	22
21	*Gdf15*	AGCTGGAACTGCGCTTACGGG	CTCCAGCCCAAGTCTTCAAGAG	28
22	*Glul*	GGAATGGAGCAGGAATATACTC	ACCGCAGTAATACGGGCCTTG	22
23	*Gstk1*	CGTACTCCTGGCTGGGGCTTTG	CAGGTGGTTGGTTGCCGCTGTC	24
24	*Gyk*	GCCTGAAACAACTGCACTAGGC	CACAGCTTTCTTCCATGTGGAG	27
25	*Hist1h1c*	CGAGCTCATCACCAAGGCTGTG	CCCTTGCTCACCAGGCTCTTC	26
26	*Hspa1b (Hsp70)*	GACAAGTCGGAGAACGTGCAG	CGAGTAGGTGGTGAAGGTCTG	25
27	*Hspb1*	CGGTGCTTCACCCGGAAATAC	GCTGACTGCGTGACTGCTTTGG	25
28	*Hspb2 (Hsp27)*	CTCACAGTGAAGACCAAGGAAG	GGATAGGGAAGAGGACACTAGG	26
29	*Hmox1*	AAGACCGCCTTCCTGCTCAAC	CGAAGTGACGCCATCTGTGAGG	28
30	*Hprt1*	CTTGCTCGAGATGTCATGAAGGAG	TAATCCAGCAGGTCAGCAAAGAAC	26
31	*Igfbp1*	GATCAGCCCATCCTGTGGAACG	TTCTCGTTGGCAGGGCTCCTTC	24
32	*Isg20l1*	TTGAAGGGCAAGGTGGTGGTG	GAGCAGGTTTGGGACATAAGTG	24
33	*Jun*	GCCAAGAACTCGGACCTTCTC	AGTGGTGATGTGCCCATTGCTG	23
34	*Kras*	GGCAAGAGCGCCTTGACGATAC	TGGTCCCTCATTGCACTGTACTCC	28
35	*Lig3*	TGCGGCTCTACTTGCCACCTTC	CATGTGTGGCTGAGCCCATGTC	27
36	*Lrp1*	GGGCCATGAATGTGGAAATTGG	GTGGCATACACTGGGTTGGTG	22
37	*Mbd1*	GGATCCTGACACTCAAGAATGG	GTTTGGGCTAACACAGGAAGAG	23
38	*Mdm2*	TTGATCCGAGCCTGGGTCTGTG	AAGATCCTGATGCGAGGGCGTC	27
39	*Myc*	B5,GTCAGCAACAACCGCAAGTGCTC	AAAGCTGCGCTTCAGCTCGTTC	32
40	*Net1*	GACCTCCACGAAGAGTGTGAAG	CTGTACACTGGAGCCACAATCC	27
41	*Pdgfb*	AAGACGCGCACAGAGGTGTTCC	GGCATTGCACATTGCGGTTATTG	33
42	*Plk2*	CTGTTGAGAGCGTCTTCAGTTG	CCATAGTTCACAGTTAAGCAGC	28
43	*Pml*	GGCAAGAAGCGTCCTTACCTTC	GGACAGCAACAGCAGTTCAGTC	28
44	*Pmm1*	TGTCCCGAGGAGGCATGATAAG	CAAAGTCATTCCCGCCAGGAC	30
45	*Ppp1r3c*	TGGAAACCTGACGGAGTGCAG	GCAAGCCTTGGACTGCCAAAG	24
46	*Rad52*	TGACGCCACTCACCAGAGGAAG	GCTGGAAGTACCGCATGCTTGG	30
47	*Rcan1*	GGTCCACGTGTGTGAGAGTG	TGGATGGGTGTGTACTCCGG	24
48	*Trp53*	TTGGACCCTGGCACCTACAATG	GCAGACAGGCTTTGCAGAATGG	26
49	*Tubb2c*	TTGGCAACAGCACCGCTATTC	TCGGACACCAGGTCGTTCATG	23
50	*Ube2e1 (UbcM3)*	AACTGGAGCCCAGCCCTAACC	TGGCCATTCTGTCGTGTTCTGC	24
51	*Ung*	AACCTGAGTGGCCTCGTCTTCC	TCTGCATCCCAGGAACCCTCTG	29

Ct values are those of the highest group in the present experimental condition.

At 28 days, three DEN-28 d-Grp-1 genes showed a dose-dependent increase of more than four-fold. Seventeen DEN-28 d-Grp-2 genes were suggested to have a gradual dose-dependent increase, though less than that for the expression in DEN-28 d-Grp-1. Ungrouped *Igfbp1* showed a dose-dependent decrease of less than 0.3-fold.

Unsupervised *k*-means clustering results are shown in Fig. 3A. Genes were classified into four clusters based on the hierarchical clustering results. Gene expression was classified into four clusters (DEN-4 h-Cluster-1 to DEN-4 h-Cluster-4) 4 h after administration, and four clusters (DEN-28 d-Cluster-1 to DEN-28 d-Cluster-4) 28 days after administration. As unsupervised *k*-means clustering was performed for 4 h and 28-day data separately, cluster member genes were different for 4 h and 28 days.

At 4 h, all 12 DEN-4 h-Cluster-1 genes exhibited a dose-dependent increase of more than eight-fold. Fourteen DEN-4 h-Cluster-2 genes showed a gradual dose-dependent increase as compared to DEN-4 h-Cluster-1 genes. Although *Myc* and *Igfbp1* in DEN-4 h-Cluster-3 had some atypical dose–response, they showed an increase of up to or greater than two-fold, as a whole. Two genes in DEN-4 h-Cluster-4 exhibited a dose-dependent decrease of less than 0.3-fold [*Cyp1a2* and *Glul*]. For 28-day data, 4 DEN-28 d-Cluster-1 genes showed a dose-dependent increase of more than two-fold. *Igfbp1* in DEN-28 d-Cluster-3 showed a dose-dependent decrease of less than 0.3-fold.

Two types of clustering results for the DEN data are summarized as follows. A total of 28 genes showed a dose-dependent increase or decrease at 4 h or 28 days after administration. Twenty-six genes in DEN-4 h-Grp-1 or DEN-4 h-Grp-2 and DEN-4 h-Cluster-1, DEN-4 h-Cluster-2 or DEN-4 h-Cluster-3 showed a dose-dependent increase ranging from 2-fold to more than 64-fold [*Bax*, *Btg2*, *Ccng1*, *Ccng2*, *Cdkn1a*, *Cyp4a10*, *Cyp21a1*, *Fos*, *Gadd45b*, *Gdf15*, *Hspb1*, *Hmox1*, *Hsp27*, *Igfbp1*, *Isg20l1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Pmm1*, *Ppp1r3c*, *Rad52*, *Rcan1* and *Tubb2c*]. Two genes in DEN-4 h-Grp-4

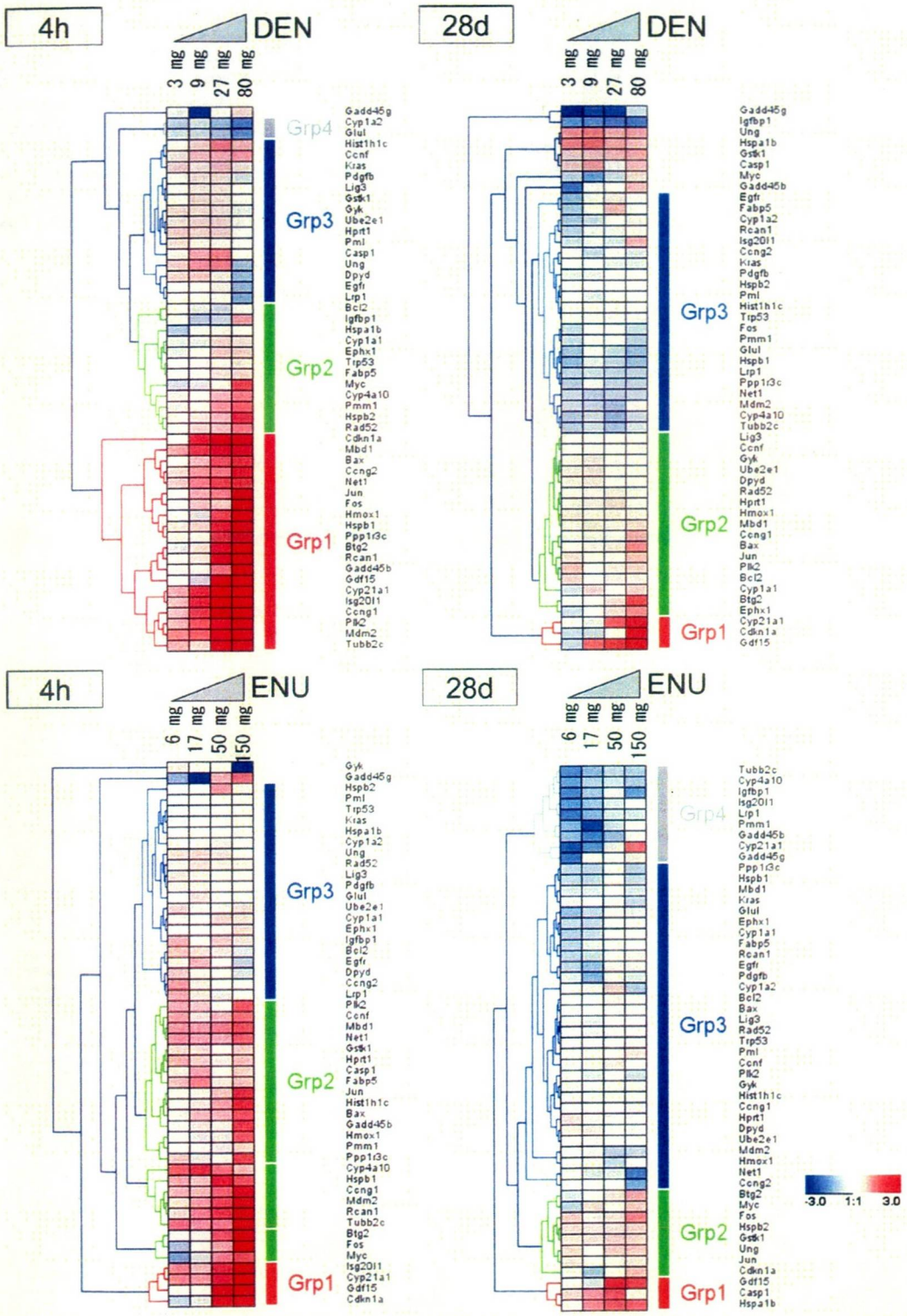

Fig. 2. Cluster analysis of gene expression after DEN and ENU treatment. The expression of 50 genes was clustered by hierarchical clustering after DEN or ENU treatment. Results of 4 h and 28 days were analyzed separately. The color displays show the $\log_2$ (expression ratio) as (1) red when the treatment sample is up-regulated relative to the control sample, (2) blue when the treatment sample is down-regulated relative to the control sample and (3) white when the $\log_2$ (expression ratio) is close to zero.

and DEN-4 h-Cluster-4 showed a dose-dependent decrease of less than 0.3-fold [*Cyp1a2* and *Glul*].

At 28 days, four genes in DEN-28 d-Grp-1 or DEN-28 d-Grp-2 and DEN-28 d-Cluster-1, which showed a dose-dependent increase at 4 h, also showed a dose-dependent increase by more than 2–4-fold [*Btg2*, *Cdkn1a*, *Cyp21a1* and *Gdf15*]. *Igfbp1* in the ungrouped group and DEN-28 d-Cluster-3 showed a dose-dependent decrease of less than 0.3-fold.

3.1.2. Identification of biologically relevant networks for DEN treatment

DEN numerical data of all 51 examined genes were analyzed by IPA, and 5 gene networks were extracted (Table 3). Five networks are also shown as bar graphs in Fig. 4.

For the 4 h time point, 35 genes were extracted in DEN-4 h-Network-1 (cancer, cell cycle and reproductive system disease); of these, 15 genes were examined in this study, and 11 of these genes showed a dose-dependent response [*Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gadd45b*, *Gdf15*, *Hspb1*, *Hspb2*, *Mdm2*, *Plk2* and *Pmm1*] (Fig. 4A, Network-1). Network-1 was a highly active network for DEN-4 h. *Trp53* and *Cdkn1a* appeared to be core genes in DEN-4 h-Network-1. *Trp53* has 15 associations [*Bax*, *Btg2*, *Casp1*, *Ccng1*, *Cdkn1a*, *Gadd45 complex*, *Gdf15*, *Hist1h1c*, *Hspb1*, *Mdm2*, *Plk2*, *Pml*, *Pmm1*, *Pdgf* complex and *Caspase* complex], and *Cdkn1a* has 9 associations [*Trp53*, *Plk2*, *Pdgf complex*, *Gdf15*, *Gadd45b*, *Gadd45g*, *Mdm2*, *Caspase* complex and *Pml*].

DEN-4 h-Network-2 (cell cycle, DNA replication, recombination, repair and cell death) consisted of 35 genes, 15 of which were examined in this study; 11 of these genes showed a dose-dependent

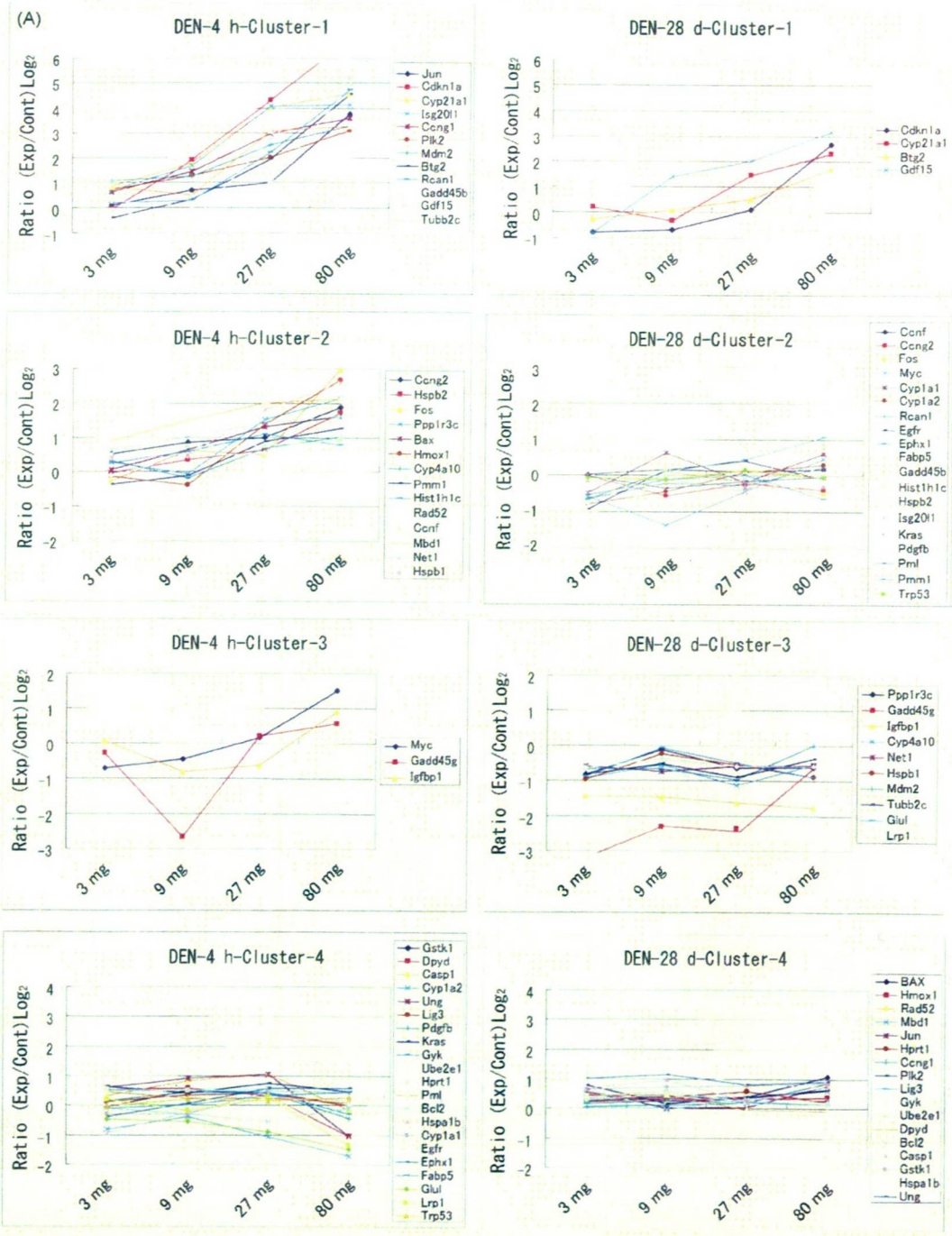

Fig. 3. Cluster analysis and dose-dependent expression pattern. The expression of 50 genes was clustered by *k*-means clustering after (A) DEN or (B) ENU treatment. Results of 4 h and 28 days were analyzed separately.

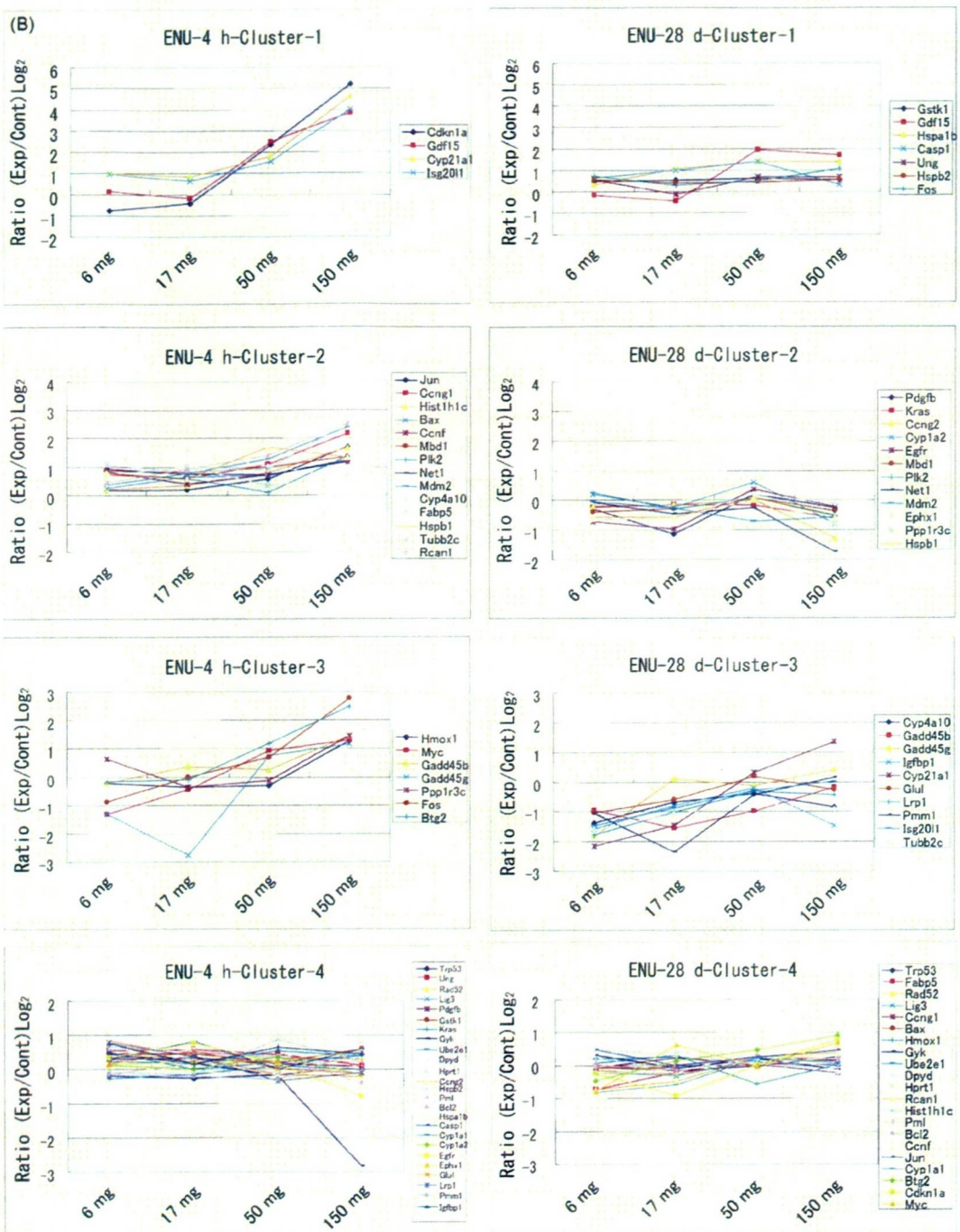

Fig. 3. (*Continued*).

response [*Ccng2*, *Cyp1a2*, *Cyp4a10*, *Cyp21a1*, *Gdf15*, *Glul*, *Igfbp1*, *Ppp1r3c Rad52*, *Rcan1* and *Tubb2c*] (Fig. 4A, Network-2). Network-2 was also a highly active network for DEN-4 h. *Il1b* and *Sp1* seemed to be core genes in DEN-4 h-Network-2. *Il1b* has five associations [*Gdf15*, *Fabp5*, *Rcan1*, *Igfbp1* and *Hprt1*], and *Sp1* has three associations [*Gdf15*, *Igfbp1* and *Cyp21a1*].

DEN-4 h-Network-3 (liver necrosis/cell death and hepatic system disease) consisted of 36 genes, 10 of which were examined in this study; 5 of these genes showed a dose-dependent response [*Fos*, *Hmox1*, *Jun*, *Myc* and *Net1*) (Fig. 4A, Network-3).

DEN-4 h-Network-4 (cell cycle, DNA replication, recombination, repair and cell death) consisted of 35 genes, 9 of which were examined in this study; 2 of these genes [*Isg20l1* and *Mbd1*] showed a dose-dependent response (Fig. 4A, Network-4).

DEN-4 h-Network-5 (cancer, drug metabolism and genetic disorder) consisted of two genes, neither of which showed a dose-dependent response in this study (Fig. 4A, Network-5).

For 28-day data, DEN-28 d-Network-1 consisted of the same genes and the same top functions as for DEN-4 h-Network-1 (Table 3(B)); however, a generally lower dose-dependent response