

結合甲基化敏感性分析和抑制性扣減雜交區分出在大腦和下視丘之間具有甲基化差異性表現的片段

張文彥、李泰林

E-mail: 9417461@mail.dyu.edu.tw

摘要

中文摘要 印記基因現象依對偶基因的親源而產生不同的epigenetic標記，藉由這些標記而區分出來自父方或是母方的對偶基因，已知可能和DNA甲基化影響核染色質結構或是和某種蛋白質與核酸之間的結合有關。在目前已知的印記基因中Igf2、Snrpn是在父源基因組上被表現，而H19、Igf2r、Mash2是在母源基因組上被表現，同時在經過追蹤嵌合鼠上細胞的表現位置後，發現父源染色體在下視丘上有專一性表現，而母源染色體在大腦上有專一性表現。有鑒於在大腦上看到此差異性的表現，我們結合了甲基化敏感性分析和抑制性扣減雜交suppression subtractive hybridization (SSH)方法來有效的找尋大腦和下視丘之間具有甲基化差異性表現的基因。目前在下視丘上找到一段有甲基化差異表現的片段落在一基因swim6之第二個內含子(intron)上，在基因庫中顯示小鼠存在兩種mRNA序列，共用exon3之後所有exon，分別為6.6 (XM_358311)及4.2 Kb (AK122528)。再進一步去確認較長的Zswim6 mRNA，發現在肝臟中表現量最多，而mKIAA1577 mRNA在8種檢測組織中mRNA的表現量相當。組織間在第二個內含子亦呈現DNA甲基化程度上顯現差異，而且以肝臟組織為最多，此現象可能與外顯子的選擇有關。 關鍵詞：印記基因，DNA甲基化，甲基化敏感性限制？，抑制性扣減雜交

關鍵詞：甲基化

目錄

目錄封面內頁 簽名頁 中文摘要.....	iii	英文摘要.....	iv
目錄.....	vi	圖目.....	vii
第一章 前言.....	xi		
1.1 研究背景.....	1	1.2 印記基因及其在生物上的能.....	1
1.1.1 研究背景.....	1	1.1.2 印記基因及其在生物上的能.....	1
1.1.3 研究背景.....	1	1.1.3 研究背景.....	1
DNA甲基轉移?作用機制.....	4	1.4 DNA甲基化和印記基因的關係.....	7
1.4 DNA甲基化和印記基因的關係.....	7	1.5 胚胎發育期間甲基化的建立過程.....	8
1.5 胚胎發育期間甲基化的建立過程.....	8	1.6 DNA甲基化與疾病.....	11
1.6 DNA甲基化與疾病.....	11	1.7 研究動機.....	11
1.7 研究動機.....	11	1.9 SWIM domain 蛋白.....	14
1.9 SWIM domain 蛋白.....	14	1.9.1 SWIM domain 與凋亡的關係.....	15
1.9.1 SWIM domain 與凋亡的關係.....	15	1.9.2 SWIM domain與轉錄默化的關係.....	17
1.9.2 SWIM domain與轉錄默化的關係.....	17	第二章 研究方法.....	27
第二章 研究方法.....	27	2.1 材料.....	27
2.1 材料.....	27	2.2 實驗方法.....	28
2.2 實驗方法.....	28	2.2.1 DNA抽取.....	28
2.2.1 DNA抽取.....	28	2.2.1.1 老鼠組織基因組的抽取.....	28
2.2.1.1 老鼠組織基因組的抽取.....	28	2.2.1.2 甲基化敏感性限制酵素反應.....	29
2.2.1.2 甲基化敏感性限制酵素反應.....	29	2.2.1.3 洋菜膠膠體電泳.....	30
2.2.1.3 洋菜膠膠體電泳.....	30	2.2.1.4 透析收集電泳凝膠中核酸片段.....	30
2.2.1.4 透析收集電泳凝膠中核酸片段.....	30	2.3 抑制性扣減雜交.....	34
2.3 抑制性扣減雜交.....	34	2.3.1 Adaptor 的製備.....	34
2.3.1 Adaptor 的製備.....	34	2.3.2 Adaptor 接合.....	34
2.3.2 Adaptor 接合.....	34	2.3.3 雜合反應.....	35
2.3.3 雜合反應.....	35	2.3.4 第一次雜合.....	35
2.3.4 第一次雜合.....	35	2.3.5 第二次雜合.....	36
2.3.5 第二次雜合.....	36	2.3.6 聚合酵素鏈鎖反應.....	36
2.3.6 聚合酵素鏈鎖反應.....	36	2.4 由洋菜膠中洗提出核酸片段.....	37
2.4 由洋菜膠中洗提出核酸片段.....	37	2.5 核酸片段選殖.....	37
2.5 核酸片段選殖.....	37	2.6 勝任細胞的製備.....	39
2.6 勝任細胞的製備.....	39	2.7 細菌的熱休克轉形作用.....	39
2.7 細菌的熱休克轉形作用.....	39	2.8 抽取細菌之質體DNA.....	40
2.8 抽取細菌之質體DNA.....	40	2.8.1 小量製備.....	40
2.8.1 小量製備.....	40	2.8.2 DNA大量製備.....	41
2.8.2 DNA大量製備.....	41	2.8.2.1 細菌菌體分解.....	41
2.8.2.1 細菌菌體分解.....	41	2.8.2.2 PEG沉澱.....	42
2.8.2.2 PEG沉澱.....	42	2.9 老鼠組織RNA萃取.....	42
2.9 老鼠組織RNA萃取.....	42	2.10 反轉錄聚合?鏈鎖反應.....	43
2.10 反轉錄聚合?鏈鎖反應.....	43	2.11 南方墨點雜交法.....	44
2.11 南方墨點雜交法.....	44	2.11.1 前雜合反應.....	44
2.11.1 前雜合反應.....	44	2.11.2 探針製備.....	45
2.11.2 探針製備.....	45	2.11.3 雜交反應與放射性偵測.....	45
2.11.3 雜交反應與放射性偵測.....	45	2.12 北方墨點雜交法.....	46
2.12 北方墨點雜交法.....	46	2.12.1 前雜合反應.....	47
2.12.1 前雜合反應.....	47	2.12.2 探針製備.....	47
2.12.2 探針製備.....	47	2.12.3 放射線顯影.....	48
2.12.3 放射線顯影.....	48	第三章 實驗結果.....	49
第三章 實驗結果.....	49	第四章 討論.....	70
第四章 討論.....	70	第五章 結論.....	75
第五章 結論.....	75	參考文獻.....	76
參考文獻.....	76	附錄.....	83
附錄.....	83	圖目錄 圖一、DNA上甲基化的鹼基結構.....	3
圖目錄 圖一、DNA上甲基化的鹼基結構.....	3	圖二、DNA甲基轉移?作用機制.....	3
圖二、DNA甲基轉移?作用機制.....	3		

.....6	圖三、 H19/Igf2基因調節機制.....9	圖四、 胚胎發育過程中甲基化的建立過程.....10
.....10	圖五、 利用LacZ為in situ marker追蹤細胞發育的位置.....18	圖六、 扣減雜交原理.....20
.....20	圖七、 抑制性扣減雜交的原理.....21	圖八、 抑制性扣減雜交的引子序列.....22
.....22	圖九、 PCR 抑制效應的原理.....23	圖十、 鋅指蛋白DNA結合位之立體圖.....24
.....24	圖十一、 不同物種間的SWI2/SNF2和MuDR蛋白質上的類SWIM domain (finger-like SWIM domain)序列和結構的保守性.....25	圖十二、 以甲基敏感性限制?區分出甲基化和沒有甲基化片段...32
.....32	圖十三、 利用電泳溶離收集瓊脂凝膠中的核酸.....33	圖十四、 T/A vector選殖位置圖.....38
.....38	圖十五、 基因組核酸之限制?分解大小分佈圖.....55	圖十六、 抑制性扣減雜交後PCR產物的電泳分析圖.....56
.....56	圖十七、 利用PCR篩選扣減出的DNA片段.....57	圖十八、 抑制性扣減雜交後PCR產物的電泳分析圖.....58
.....58	圖十九、 利用PCR篩選扣減出的DNA片段.....59	圖二十、 利用南方氏點墨法確認Ha2在cortex及hypothalamus限制?消化後差異性片段.....60
.....60	圖二十一、 利用南方氏點墨法確認Cb3在cortex及hypothalamus限制?消化後差異性片段.....61	圖二十二、 8個組織中的Zswim6的差異性表現.....64
.....64	圖二十三、 8個組織中的mKIAA1577的差異性表現.....66	圖二十四、 組織差異性DNA甲基化於Zswim6區域.....68
.....68	圖二十五、 利用AP-PCR找尋小鼠大腦及下視丘DNA甲基化差異性片段.....74	表目錄 表一、 Ag、 Gg胚胎與腦部發育的比較.....19
.....19	表二、 第一次抑制性扣減雜交後8個甲基化差異性片段.....62	表三、 第二次抑制性扣減雜交後2個甲基化差異性片段.....63

參考文獻

- 參考文獻 鄭又璋, 2004, 利用甲基化核糖核酸多形分析法找尋父母源標記基因, 私立大葉大學分子生物科技研究所, 碩士論文。
- Antequera, F. & Bird, A. (1993). Number of CpG islands and genes in human and mouse. *Proc. Natl. Acad. Sci. U S A* 90, 11995-11999.
- Bellefroid, E.J., Lecocq, P.J., Benhida, A., Poncelet, D.A., Belayew, A. & Martial, J.A. (1989). The human genome contains hundreds of genes coding for finger proteins of the Kruppel type. *Dna* 8, 377-387.
- Bestor, T.H. (2000). The DNA methyltransferases of mammals. *Hum. Mol. Genet.* 9, 2395-2402.
- Bestor, T.H. (1992). Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.* 11, 2611-2617.
- Bestor, T.H., Laudano, A.P., Mattaliano, R. & Ingram, V.M. (1988). Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.* 203, 971-983.
- Bird, A.P. & Southern, E.M. (1978). Use of restriction enzymes to study eukaryotic DNA methylation: I. The methylation pattern in ribosomal DNA from *Xenopus laevis*. *J. Mol. Biol.* 118, 27-47.
- Byers, R.J., Hoyland, J.A., Dixon, J. & Freemont, A.J. (2000). Subtractive hybridization-genetic takeaways and the search for meaning. *Int. J. Exp. Pathol.* 81, 391-404.
- Choo, Y. & Klug, A. (1994). Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. *Proc. Natl. Acad. Sci. USA* 91, 11168-11172.
- Constancia, M., Pickard, B., Kelsey, G. & Reik, W. (1998). Imprinting mechanisms. *Genome Res.* 8, 881-900.
- Denissenko, M.F., Chen, J.X., Tang, M.S. & Pfeifer, G.P. (1997). Cytosine methylation determines hot spots of DNA damage in the human P53 gene. *Proc. Natl. Acad. Sci. U S A* 94, 3893-3898.
- Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. & Siebert, P.D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. U S A* 93, 6025-6030.
- Filippova, G.N., Qi, C.F., Ulmer, J.E., Moore, J.M., Ward, M.D., Hu, Y.J., Loukinov, D.I., Pugacheva, E.M., Klenova, E.M., Grundy, P.E., Feinberg, A.P., AM C.J., Moerland, E.W., Cornelisse, C.J., Suzuki, H., Komiya, A., Lindblom, A., F D.B., Neiman, P.E., Morse, H.C. 3rd., Collins, S.J. & Lobanenkov, V.V. (2002). Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter its DNA-binding specificity. *Cancer Res.* 62, 48-52.
- Hagemann, C. & Blank, J.L. (2001). The ups and downs of MEK kinase interactions. *Cell Signal* 13, 863-875.
- Holliday, R. & Pugh, J.E. (1975). DNA modification mechanisms and gene activity during development. *Science* 87, 226-232.
- Hoovers, J.M., Mannens, M., John, R., Blik, J., van Heyningen, V., Poreeous, D.J., Leschot, N.J., Westerveld, A. & Little, P.F. (1992). High-resolution localization of 69 potential human zinc finger protein gene: a number are clustered. *Genomics* 12, 254-263.
- Imamura, T., Ohgane, J., Ito, S., Ogawa, T., Hattori, N., Tanaka, S. & Shiota, K. (2001). CpG island of rat sphingosine kinase-1 gene: tissue-dependent DNA methylation status and multiple alternative first exons. *Genomics* 76, 117-125.
- Issa, J.P., Vertino, P.M., Wu, J., Sazawal, S., Celano, P., Nelkin, B.D., Hamilton, S.R. & Baylin, S.B. (1993). Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J. Natl. Cancer Inst.* 85, 1235-1240.
- Jacobs, G. H. (1992). Determination of the base recognition position of zinc fingers from sequence analysis. *EMBO J.* 11, 4507-4517.
- Jeffrey, R.M. (2001). Imprinting in the germ line. *Stem Cells* 19, 287-294.
- Jeltsch, A. (2002). Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. *Chem.Bio.Chem.* 3, 274-293.
- Kaffer, R., Gringerg, A. & Pfeifer, K. (2001). Regulatory mechanisms at the mouse Igf2/H19 locus. *Mol. Cell Biol.* 21, 8189-8196.
- Kautiainen, T.L. & Jones, P.A. (1986). DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. *J. Biol. Chem.* 261, 1594-1598.
- Keverne, E.B., Fundele, R., Narasimha, M., Barton, S.C. & Surani, M.A. (1996). Genomic imprinting and the differential roles of parental genomes in brain development. *Devel. Brain Res.* 92, 91-100.
- Laird, P.W. & Jaenish, R. (1996). The role of DNA methylation in cancer genetics and epigenetics. *Ann. Rev. Genet* 30, 441-464.
- Laity, J.H., Lee, B.M. & Wright, P.E. (2001). Zinc finger proteins: new insights into

structural and functional diversity. *Curr. Opin. Struct. Biol.* 11, 39-46. Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R. & Li, E. (1996). De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* 122, 3195-3205. Lui, K., Wang, Y., Cantemir, C. & Muller, T. (2003). Endogenous assay of DNA methyltransferase: evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. *Mol. Cell Biol.* 23, 2709-2719. Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998). Cytosolic translocation required for MEKK-1 induced apoptosis. *Cell* 94, 481-490. Macleod, D., Ali, R.R. & Bird, A. (1998). An alternative promoter in the mouse major histocompatibility complex class II I-Abeta gene: implications for the origin of CpG islands. *Mol. Cell Biol.* 18, 4433-4443. Makarova, K.S., Aravind, L. & Koonin, E.V. (2002). SWIM, a novel Zn-chelating domain present in bacteria, archaea and eukaryotes. *Trends Biochem Sci.* 27, 384-386. Mann, J.R. (2001). Imprinting in the germ line. *Stem Cells* 19, 287-294. McGrath, J. & Solter, D. (1984). Completion of mouse embryo genesis requires both the maternal and paternal genomes. *Cell* 37, 179-183. Okano, M., Bell, D. W., Haber, D. A. & Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247-257. Okano, M., Xie, S. & Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA(cytosine-5)methyltransferases. *Nat. Genet.* 19, 219-220. Pfeifer, K. (2000). Mechanisms of genomic imprinting. *Am. J. Hum. Genet* 67, 777-787. Reik, W. & Walter, J. (2001). Genomic imprinting: parental influence on the genome. *Nature* 2, 21-32. Reinhart, B., Eljanne, M. & Richard, J. (2002). Shared role for differentially methylated domains of imprinted gene. *Mol. Cell Biol.* 22, 2089-2098. Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Langeur, C., Kinzler, K.W., Baylin, S.B. & Vogelstein, B. (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cell. *Nature* 416, 552-556. Rice, J.C., Massey-Brown, K.S. & Futscher, B.W. (1998). Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. *Oncogene* 17, 1807-1812. Schlesinger, T.K., Bonvin, C., Jarpe, M.B., Fanger, G.R., Cardinaux, J.R., Johnson, G.L. & Widmann, C. (2002). Apoptosis stimulated by the 91kDa caspase cleavage MEKK1 fragment requires translocation to soluble cellular compartments. *J. Biol. Chem.* 277, 10283-10291. Singer-Sam, J., Grant, M., LeBon, J.M., Okuyama, K., Chapman, V., Monk, M. & Riggs, A.D. (1990). Use of a HpaII-polymerase chain reaction assay to study DNA methylation in Pcg-1 CpG island of mouse embryos at the time of X-chromosome inactivation. *Mol. Cell Biol.* 10, 4987-4989. Srivastava, M., Hsien, A. & Pfeifer, K. (2000). H19 and Igf2 monoallelic expression is regulated in two distinct way by a shared cis acting element. *Genes* 14, 1186-1195. Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. & Cedar, H. (1982). Clonal inheritance of the pattern of DNA methylation in mouse cells. *Proc. Natl. Acad. Sci. U S A* 79, 61-65. Vanaja, D.K., Cheville, J.C., Iturria, S.J. & Young, C.Y. (2003). Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res.* 63, 3877-82. Wigler, M., Levy, D. & Perucho, M. (1981). The somatic replication of DNA methylation. *Cell* 24, 33-40. Yoder, J.A., Walsh, C.P. & Bestor, T.H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 13, 335-340.