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Table 1. Allele frequencies of 5 SNPs of the FOXP2 gene in autism patients and controls

			Patients				Controls					
			Allele A		Allele B		Allele A		Allele B			
locus	db SNP ID	Allele A / B	N	%	N	%	N	%	N	%	chi-square	p-value
SNP 1	rs2106900	[C/T]	234	70%	102	30%	294	70%	124	30%	0.043	0.837
SNP 2	rs2061183	[C/G]	84	25%	254	75%	105	25%	313	75%	0.007	0.933
SNP 3	rs1456029	[A/G]	195	58%	143	42%	221	52%	203	48%	2.354	0.125
SNP 4	rs1005958	[A/G]	309	92%	27	8%	393	93%	31	7%	0.140	0.709
SNP 5	rs1058335	[C/T]	224	66%	114	34%	266	63%	156	37%	0.860	0.354

The SNPs 1-5 are located at introns 1, 2 7, 14 and the 3' region of the gene, respectively.

Table 2. Allele frequencies of 7 SNPs of the *PTPRZ1* gene in autism patients and controls

			Patients				Controls					
			Allele A		Allele B		Allele A		Allele B			
locus	db SNP ID	Allele A / B	N	%	N	%	N	%	N	%	chi-square	p-value
SNP 1	rs740960	[G/T]	223	66%	113	34%	277	66%	143	34%	0.014	0.904
SNP 2	rs1206504	[A/G]	234	70%	102	30%	276	66%	144	34%	1.312	0.252
SNP 3	rs1196490	[A/G]	112	33%	226	67%	153	36%	271	64%	0.721	0.396
SNP 4	rs1196509	[C/T]	318	94%	20	6%	400	94%	26	6%	0.012	0.914
SNP 5	rs1196475	[C/T]	217	65%	119	35%	284	67%	142	33%	0.362	0.547
SNP 6	rs1147489	[A/G]	276	82%	62	18%	357	84%	67	16%	0.864	0.353
SNP 7	rs1206381	[A/C]	223	67%	111	33%	290	69%	128	31%	0.584	0.445

The SNPs 1-7 are located at introns 1 (SNPs 1 and 2), 2, 4, 11, 19 and 27, respectively.

Table 3. The strength of LD (denoted as D') between pairs of SNPs of (a) *FOXP2* and (b) *PTPRZ1*in autism patients (the lower diagonal) and controls (the upper diagonal)

(a) *FOXP2*

SNP	1	2	3	4	5
1		0.94	0.37	0.35	0.14
2	0.92		0.40	0.47	0.82
3	0.53	0.43		0.21	0.22
4	0.59	0.29	0.88		0.74
5	0.15	0.54	0.09	1.00	

(b) *PTPRZ1*

SNP	1	2	3	4	5	6	7
1		0.40	0.91	0.24	0.53	0.50	0.56
2	0.62		0.44	1.00	0.21	0.34	0.24
3	0.90	0.67		0.26	0.59	0.55	0.61
4	0.15	0.09	0.28		1.00	1.00	1.00
5	0.67	0.52	0.85	1.00		1.00	0.99
6	0.66	0.52	0.85	1.00	1.00		1.00
7	0.67	0.56	0.85	1.00	1.00	1.00	

特集 脳を育む

発達障害の分子基盤

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KEY WORDS

・autism
・a genome-wide screen
・chromosome
・15q11-13
・7q21-33

SUMMARY

自閉症ならびにそのスペクトラム障害を例に，発達障害に関連する可能性の高い遺伝子について概観する．自閉症圏の疾患では，連鎖解析ならびに染色体の解析から7q，15qなどの染色体領域に関連遺伝子の位置することが示唆されている．これらの領域はimprinting遺伝子を多く含むなど，遺伝学的にも興味深い領域である．それらの領域で脳の発生・発達に関与する遺伝子が，まず候補遺伝子として注目される．また自閉症をはじめ多くの発達障害で発病の性差が観察されるが，脆弱X症候群のFMR-1や脆弱X症候群EのFMR-2，Rett症候群のMECP 2，神経線維症腫のNF 1など，多くのX染色体上の遺伝子も発達障害にかかわることがこれまで明らかにされている．

はじめに

本稿では発達障害の代表として自閉症を取り上げ，その発病に関連する遺伝子・染色体領域の候補について解説する．

自閉症は，社会的相互的交渉の質的異常，コミュニケーションの質的異常，限局した興味や行動のパターンという行動的症候，および乳幼児期からこれらの症候が明らかになるという経過を特徴とする発達障害と定義される．自閉症の一般人口における有病率は，最近の報告によると狭義の自閉症で17～40/10,000，自閉性障害全体では60/10,000に及ぶとされている[1]．性差として男性に多い（男女比＝約4：1）．合併が多く認められる疾患としては結節性硬化症[2]や脆弱X症候群[3][4]，神経線維症腫があげられる．患者同胞での発病率は約4%である．双生児での発病一致率は一卵性では60～80%であるのに対して，二卵性では5%弱と同胞での発病率とほとんど違いがない[5][6]．一方，近年は軽症例を含めた広い範囲での診断のおこなわれることが増えているが，軽症例まで含めた拡大表現型での双生児の発病一致率は，一卵性で80～90%，二卵性では10～30%と報告されている[5][7]～[9]．いずれにしても，双生児での発病一致率は二卵性にくらべて一卵性ではるかに高く，自閉症の発症には遺伝的要因の影響がきわめて強く，また複数の遺伝子が関与している可能性の高いことを示唆している．

1．連鎖研究と染色体研究で示唆される染色体領域

わが国での連鎖解析は残念ながらこれまで皆無であるが，海外では全ゲノムでの連鎖研究が複数発表されている（**表1**）[10]～[16]．他の複雑疾患同様，決定的なロッド値は得られてはいないが，複数の研究で共通して示唆され

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表 1. ゲノムワイド連鎖解析の結果

研究グループ	年	兄弟ペア数	染色体領域	マーカー	最大のLOD値
IMGSAC[10]	1998	87	7q 16p 4p	D7S530/D7S684 D16S407/D16S3114 D4S412	MLS 2.53 MLS 1.51 MLS 1.55
CLSA[11]	1999	75	13q 13q 7q	D13S217/D13S1229 D13S800 D7S1813	MMLS/het 2.3 MMLS/het 3.0 MMLS 2.2
Philippe *et al*[12]	1999	51	6q	D6S283	MMLS 2.23
Risch *et al*[13]	1999	147	1p	D1S1675	MMLS 2.15
Liu *et al*[14]	2001	総数 118 狭義 75	5q Xqter 19p Xqter 16p 19q 5q	D5S2494 DXS1047 D19S714 DXS1047 D16S2619 D19S587/D19S601 D5S2488	MMLS 2.55 X-MLS 2.56 MMLS 2.53 X-MLS 2.67 MMLS 1.93 MMLS 1.70 MMLS 1.63
IMGSAC[15]	2001	総数 152 狭義 127	2q 7q 16p 17q 2q	D2S2188 D7S477 D16S3102 HTTINT2 D2S2188	MMLS 3.74 MMLS 3.20 MMLS 2.93 MMLS 2.34 MMLS 4.80
Shao *et al*[16]	2002	96	2q 3p 7q 15q 19q Xq	D2S116 D3S3680 D7S495 D15S659 D19S425 DXS6789	MLS 1.30 MLS 1.51 MLS 1.66 MLS 0.96 MLS 1.21 MLS 2.54

MLS：Maxium Lod Score　MMLS：Multipoint MLS　（筆者作成）

ている染色体領域がみられる．おもな領域としては2番染色体長腕，7番染色体長腕，16番染色体短腕があげられる．染色体異常との関連で最も注目されている領域は，15番染色体長腕領域（15q11-13）である．15q領域は自閉性障害の染色体異常において最も報告が多く，自閉症の1～3%にこの領域の異常が認められている．最も多くみられる異常は母方由来の重複で[17]，中心体から15qの近位部が2量体となり，重複した染色体が付加したisodicentric chromosome 15〔idic（15）〕などがみられる．この染色体領域の欠損による疾患として知られるのが，父方由来の欠損によるPrader-Willi症候群と母方由来の欠損によるAngelman症候群である．このうちAngelman症候群では自閉症例が報告されており，重複の例と同様imprinting遺伝子（この場合は母方由来が発現する遺伝子）が自閉症発症に関与する可能性を示している．

これらの染色体領域のうち，7q21-33近傍，15q11-13領域を中心に，候補遺伝子の例を以下にあげる．またこれらの常染色体のほかに，発病の性差から注目されるのがX染色体である．すでに自閉症以外の多くの発達障害の原因遺伝子がX染色体上に同定されているが，それらを含むいくつかの遺伝子についても説明を加えたい．

1）7番染色体

連鎖解析から注目される領域は，7q21-33近傍があげられる．7q31領域においては「言葉」に関連するSPCH1領域が明らかにされ，この領域からグルタミンのくり返し配列をもつFOXP2遺伝子が単離され

た[16)19)]．さらに，脳の形成に関連するWNT 2遺伝子，セロトニン受容体5-HT 2 A遺伝子，Reelin遺伝子も7番染色体上に存在している．Reelin，WNT 2遺伝子は脳の発生に関係する遺伝子として，自閉症との関連可能性においても注目されている[20)21)]．

A．FOXP 2 (forkhead box proteins P 2)

FOXP 2は読字障害家系および孤発例患者で報告され，読字障害が連鎖すると（SPCH 1）報告されていた7 q 31領域に局在する．FOXP 2はforkhead DNA-binding domainをもつ転写因子である．自閉症では言語障害が主要な症状であり，FOX 2も自閉症の候補遺伝子として期待されたが，自閉症との有意な関連は確認されていない[22)]．

B．WNT 2 (wingless-type MMTV integration site family member 2)

7 q 31-33に存在する．WNTは中枢神経を含む多くの臓器で発生，分化に関与する．WNT 2では，自閉症2家系において各々Arg 299 Trpと Leu 5 Arg変異が検出されたが[21)]，その後の研究では自閉症との関連は確認されなかった[23)]．

C．RAY 1/ST 7 (homo sapiens suppression of tumorigenicity 7)

7 q 31に位置し，かつ7 q 31.3と13 q 21のあいだの転座（7；13）（q 31.3；q 21）を有する自閉症患者の転座の切断点に同定された遺伝子である[19)]．役割としては腫瘍抑制遺伝子（tumor-suppressor gene）と考えられており，これまでのところ変異のスクリーニングでは大勢の自閉症全般との関連の証拠は得られていないが，今後さらに検討が必要である．

D．Reelin

7 q 22に存在し，中枢神経系の層構造形成に重要な遺伝子であり，小脳低形成の病因遺伝子となる．Reelin遺伝子は，開始コドンATGのすぐ5′側にあるATGくり返し多型が自閉症に連鎖するとの報告がある[20)]．また，自閉症患者の小脳でReelin遺伝子の発現が低下しているとの報告もある[24)]．しかしその後，関連を否定する報告もあり[25)]，さらに検討が必要である．

2）15番染色体

15 q 11-13領域上で最初に自閉症発症の候補遺伝子と推定されたのが，GABRB 3（γ-aminobutyric acid type-A receptor beta 3 subunit）遺伝子である[26)]．GABRB 3遺伝子は$GABA_A$受容体サブユニットをコードしており，GABA系が発達早期から発現していることから，自閉症の感受性遺伝子ではないかと期待された．しかしその後の研究では否定的な報告がなされている[27)]．15 q 11-13領域上の遺伝子で他に候補遺伝子となったのがUBE 3 A（ubiquitin protein ligase E 3 A）遺伝子である．UBE 3 Aは主として脳に発現し，Angelman症候群の原因遺伝子とされている．Angelman症候群で自閉症例が報告されることから，自閉症の感受性遺伝子であることが示唆され検討されているが，結論は得られていない[28)]．

3）X染色体

A．FMR-1遺伝子（脆弱X症候群・FRAX）

FMR-1（fragile X mental retardation protein 1）は脆弱X（fragile X syndrome：FRAX）症候群の原因遺伝子である．最近U. S. Center for Control（CDC, July 2001）がまとめた結果によると，一般人口における本症の発病は男性で1/4000～6000，女性で1/8000～9000である．X連鎖性の家族性精神遅滞のうち，20～40%は本症によると考えられる．この疾患は精神遅滞が中心であるが，自閉的な傾向をもつため，自閉症との関連についても研究がされた．実際に，幼児期の脆弱X症候群患児では，15～33%がDSM-IVの自閉症診断カテゴリーにあてはまるといわれている．FMR-1遺伝子は，Xq 27.3に位置し，その脆弱部位（FRAXA）に存在するCGCリピート配列の異常延長によって発症する．FMR-1蛋白はさまざまなmRNAと結合し，ニューロンにおける特定の遺伝子群の発現を調節する役割があるのではないかと考えられている[29)]．FMR-1遺伝子と自閉症との関係については，関連研究で否定的な報告が多く，当初考えられていたほどではない可能性も高いが，FMR-1蛋白（FMRP）発現量との関連など，より詳細な観点での研究の予知が残されている．

B．FMR-2遺伝子（脆弱X症候群E）

脆弱X症候群の多くはFMR1遺伝子異常によって発症するが，1993年にFMR1の近傍X28の領域から新たな遺伝子FMR2が発見された．この遺伝子は5' UTRのCCGリピート異常延長により遺伝子発現が消失し発症する．身体的特徴はなく，軽度の精神遅滞や学習障害のみの例が多い．発現頻度は低く，現時点においてわが国での報告はない．FMR-2蛋白は核内に存在し，転写を促進する働きがあることが示唆されている．本遺伝子は胎児期，脳全体に発現しているが，生後は海馬，扁桃体と大脳皮質の一部に発現が限局している[30]．

C．MECP2遺伝子（Rett症候群）

MECP2（methyl-CpG-binding protein 2：MeCP2）はメチルCpG結合蛋白2をコードする遺伝子で，Xq28に位置し，その変異はRett症候群の原因となる[31]．

Rett症候群は女性に発症する疾患として知られていたが，近年，男性の精神遅滞患者のなかにもMECP2遺伝子の異常を示す例が発見されている[32]．自閉症との関連では一例の報告があるのみで[33]，自閉症全体との関連はあまり大きくないかもしれない[34]．

D．NF1遺伝子（神経線維腫症，neurofibromatosis type 1）

自閉症における神経線維腫症の有病率は，一般人口の150倍と高値を呈する．NF1遺伝子上のマーカーであるGXAluと重度の自閉症との関連が報告されたが[35]，その後の研究結果は対象とする民族の違いもあってかさまざまで[36][37]，さらに検討が必要である．

E．NLGN3，NLGN4遺伝子（neuroligin type 3, 4）

Neuroligin（NLGN）はシナプスにおけるニューロン間の結合に重要な役割を果たす．最近，NLGN3遺伝子（Xq13.1）のmissense変異とNLGN4遺伝子（Xp22.33）のnonsense変異が，自閉症圏の罹患同胞対（NLGN4の変異では同胞対とその母親）で報告されている[38]．自閉症全体とのかかわりは不明であるが，NLGNの中枢神経における役割から考えると興味深い．

2．その他の遺伝子について

1）セロトニン関連遺伝子

自閉症患者において血中あるいは髄液中の5-HTレベルが高いこと[39]，また，自閉症の脳内におけるセロトニン代謝異常の報告，5-HT再取り込み阻害薬（selective serotonin reuptake inhibitors：SSRI）が自閉症患者の症状の一部改善に有効である事実から，セロトニン関連遺伝子に注目した研究も多い．当初，Cookら[40]はセロトニントランスポーター（5-HTT）遺伝子のプロモータ領域多型が自閉症と関連する可能性を報告したが，その後多くの研究においてその結果は否定されている[41]~[45]．しかし他の多型を追加して検討することで，有意な関連が認められたとの報告もある[46]．血中セロトニン濃度と同遺伝子との関連については否定的な報告がされている[47]．しかし最近，この関係について関連性を指摘する報告もある[48]．5-HT2C受容体遺伝子は，自閉症との連鎖の可能性があるXq24に位置しており，興味がもたれる[49]．

2）HOXA1，HOXB1遺伝子（homeo box A1, B1）

HOXA1，B1はともに頭部の発生にかかわる遺伝子である．Ingramら[50]はHoxA1遺伝子のノックアウトマウスにおいて，患者の脳幹病理との類似，サリドマイド児と同様な四肢および耳の奇形，などを観察し，これらの遺伝子の自閉症への関与の可能性を指摘した．実際の遺伝子関連研究では十分な関連は確認されていない[51]．しかし最近，HOXA1 A218Gの遺伝的多型と自閉症患者の頭囲の増大との関連が報告されている[52]．

おわりに

発達障害の代表として自閉症をとりあげ，候補遺伝子について解説した．現在の段階では自閉症の疾患感受性遺伝子の特定には至っていないが，ゲノムワイド連鎖研究その他の結果，すでにいつかの染色体領域が自閉症の候補遺伝子として示唆されており，それぞれの領域での検討が進められている．今後の報告が期待される．

❖❖❖

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Coordinate Downregulation of a Novel Imprinted Transcript *ITUP1* with *PEG3* in Glioma Cell Lines

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Abstract

The human paternally expressed gene 3 (*PEG3*) on chromosome 19q13.4 is one of the candidate tumor suppressor genes for glioma. We have previously reported that the epigenetic silencing of *PEG3* expression in glioma cell lines is dependent on aberrant DNA methylation of an exonic CpG island. Here, we have identified three expressed sequence tags (ESTs), H80201, H78825 and AW197312, that exhibit paternal allele-specific expression, using human monochromosomal hybrids containing the paternal or maternal origin of *PEG3* locus. The EST H80201 was shown to be expressed only from the paternal allele in normal human lymphoblasts by utilizing a single nucleotide polymorphism (SNP). Monoallelic expression of EST H80201 was also detected in non-tumor adult human brain tissues of gliomas. These ESTs were located directly adjacent to *PEG3* in a head-to-head orientation. We have named this new transcript, imprinted transcript 1, which is located upstream but oppositely oriented to *PEG3* (*ITUP1*). The *ITUP1* showed a similar expression profile with *PEG3* in glioma cell lines. Bisulfite genomic sequencing and reverse transcription (RT)-PCR analysis indicated that hypermethylation of the promoter region correlated with the absence of these transcripts. This suggests that *ITUP1* and *PEG3* are coordinately regulated, and that downregulation of the both genes may be important in the development of glioma.

Key words: Genomic imprinting; glioma; Chromosome 19q; CpG island; methylation; tumor suppressor gene

1. Introduction

Genomic imprinting, the phenomenon whereby monoallelic expression of certain genes occurs depending on their parental origin, plays an important role in mammalian development, growth and cell differentiation.[1,2] Disturbance of normal imprinting leads to abnormal embryogenesis, some inherited diseases and is also associated with various types of cancer.[3–5]

The *PEG3* gene is imprinted and localized to 19q13.4, which encodes a Krüppel-type (C2H2) zinc-finger protein, is expressed in the brain, ovary, testis and placenta in adult tissues,[6] and possesses tumor-suppressing activity in glioma cell lines.[7] We have previously reported that the epigenetic silencing of *PEG3* expression in glioma cell lines depends on aberrant DNA methylation of an exonic CpG island.[8]

Most imprinted genes are clustered in chromosomal domains, indicating that genomic imprinting is a long-range phenomenon that affects relatively large chromosomal regions. The human homologue of ubiquitin-specific processing protease 29 gene (*Usp29*), a paternally expressed mouse-imprinted gene, has also been mapped on human chromosome 19q13.4 in the opposite direction to that of *PEG3*. The genomic distance between *USP29* and *PEG3* is estimated to be 3.5 Mb.[9] Analysis of the *USP29* cDNA sequence revealed a predicted protein consisting of a ubiquitin carboxyl-terminal hydrolase domain. *USP29* may be involved in the ubiquitin pathway like the candidate Angelman syndrome

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gene, ubiquitin ligase (*UBE3A*).[10] The isolation of the maternally expressed gene, imprinted zinc-finger gene 3 (*ZIM3*) and paternally expressed gene, zinc-finger gene 264 (*ZNF264*), adjacent to *PEG3*, suggests the presence of a large imprinted domain in this region.[11]

To develop an *in vitro* assay system for the investigation of imprinted loci in humans, we have previously established a library of mouse A9 hybrids containing a single human chromosome of defined parental origin, via microcell-mediated chromosome transfer (MMCT).[12] The imprinting status of the transferred human chromosomes was maintained faithfully in mouse A9 hybrids, for example, the appropriate imprint-dependent expression, allele-specific DNA methylation and histone H4 acetylation of human imprinted genes.[13–17] Using this *in vitro* assay system, we have screened differentially expressed transcripts and differentially methylated CpG islands localized to the *PEG3* region to identify additional imprinted genes.

Here we identified partial sequences of a novel imprinted transcript named *ITUP1* in the *PEG3* imprinted domain, and demonstrated that the DNA methylation-dependent epigenetic silencing of the same CpG islands resulted in similar expression profiles of *ITUP1* and *PEG3* in glioma cell lines.

2. Materials and Methods

2.1. Cell lines and tissues

Human fibroblasts, isolated from a normal adult male, were established by the standard method, and were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum. These human fibroblasts were used as donor cells to construct three separate mouse A9 hybrid clones containing a paternal or maternal human chromosome 19 tagged with pGK*neo*, as described in Inoue et al.[18] These hybrid cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 800 μg/ml GENETICIN (Gibco BRL, Rockville, MD, USA) and maintained in an incubator set at 37°C with 5% CO_2. Mouse A9 cells are derivatives from mouse L cells, and were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% FCS. Epstein-Barr virus (EBV)-transformed lymphoblast cultures were obtained using standard procedures and were maintained at 37°C with 5% CO_2 in RPMI 1640 (Gibco BRL) supplemented with 10% FCS. Human glioma cell lines (HTB-12, HTB-13, HTB-14, HTB-16, HTB-17, HTB-138, CRL-1620 and CRL-2020) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The U-251MG cell line, human glioma cell line, was provided by Dr. Kamitani. These cells were cultured at 37°C with 5% CO_2, in RPMI 1640 (Gibco BRL) supplemented with 10% FCS.

Non-tumor brain tissues were used for extraction of total RNA and genomic DNA. All samples were obtained from Tottori University Hospital with informed consent and acquired with the approval of the Ethical Committee of the Faculty of Medicine, Tottori University.

2.2. Treatment of cells with 5-aza-2′-deoxycytidine and/or trichostatin A

Human glioma cell lines were plated at a density of 5×10^5 cells/dish. After 24 h, 1 μM of 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma) or the same volume of ethanol as a control was added to the medium. The culture medium was changed every two days. Five days after starting the treatment of 5-aza-dC or ethanol, 5-aza-dC with or without 100 ng/ml of trichostatin A (TSA) (Wako Pure Chemicals, Osaka, Japan) were added to the medium. All the cells were harvested and analyzed after 24 h.

2.3. Mapping of ESTs and CpG islands

DNA and EST database searches were performed using the BLAST programs on the NCBI server (http://www.ncbi.nlm.nih.gov/). Genomic sequences from the *PEG3* locus were filtered for human repetitive elements using RepeatMasker (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). The cDNA clones corresponding to human ESTs were assembled with the ESTblast programs (http://www.hgmp.mrc.ac.uk/ESTBlast/). After correcting sequence ambiguities that were present in the human ESTs, PCR primers were synthesized and used to amplify cDNA fragments. CpG islands were identified on the *PEG3* locus using the GRAIL program (http://compbio.ornl.gov/Grail-1.3/).

2.4. ESTs screen

Total RNA was extracted from frozen A9 hybrid cells by guanidinium isothiocyanate extraction. Prior to reverse transcription, total RNA was treated with RNase-free DNase I (NIPPON GENE, Tokyo, Japan). To investigate the possibility of DNA contamination, first strand cDNA synthesis was performed with (RT+) or without (RT−) M-MLV reverse transcriptase (Gibco BRL) using an oligo $(dT)_{15}$ primer (Promega, Madison, WI, USA). To confirm the synthesis of first strand cDNA, the expression of mouse and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was detected by PCR primers RPC1 (5′CCATCTTCCAGGAGCGAGA-3′) and RPC2 (5′-TGTCATACCAG GAAATGAGC-3′). PCR conditions were 20 cycles of 94°C for 45 s, 58°C for 30 s and 72°C for 90 s.

RT-PCR was performed using the previously generated cDNA with *Taq* GOLD polymerase (Perkin Elmer, Foster, CA, USA) using a step down protocol. Primer sequences for 9 ESTs on the *PEG3* locus and reaction

conditions are available upon request. In general, the reaction parameters were as follows: 95°C initial denaturation for 10 min, 95°C for 30 s, 60°C annealing for 30 s, 72°C for 30 s. Nine rounds of PCR were carried out in this manner, with the annealing temperature being reduced by 2°C every three cycles to a final annealing temperature of 56°C. Twenty-four subsequent rounds of PCR were carried out as follows: 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, followed by a 5 min final extension at 72°C. Amplified fragments were resolved on 2% agarose gels followed by staining with SYBR Green I (Molecular Probes, Eugene, OR, USA).

2.5. *Screening of differential methylated regions (DMRs)*

The methylation status of the 7 CpG islands on the *PEG3* locus were assessed by a methylation-sensitive PCR assay. Genomic DNA extracted from A9 hybrids was digested with *HhaI* (NIPPON GENE), *HpaII* (Takara, Kyoto, Japan) or *MspI* (NIPPON GENE) prior to PCR amplification. Digested or undigested genomic DNA samples were subjected to PCR in the presence of GC Melt (Clontech, Palo Alto, CA, USA) using primers flanking the restriction sites. Primer sequences for the 7 CpG islands and each reaction condition are available upon request. Amplified fragments were resolved on 2% agarose gels followed by staining with ethidium bromide to test for the presence or absence of a post-digestion PCR product.

2.6. *Analysis of gene expression*

The cDNA samples from non-tumor human brain tissues, human glioma cell lines and lymphoblasts were amplified using the primers and amplification conditions as described above. As a control, integrity of RNA was assessed by amplification of mouse and human *GAPDH* genes as described above. The primers for imprinting (or monoallelic expression) analysis using an A/C polymorphism in EST H80201 were 16-2F (5′-TGTGGTTAATTACCAAAAGGTGTTT-3′) and 16-2R (5′-TCAGGGTACTACCACATTCACC-3′). RT-PCR and sequencing of PCR products were used to analyze allele-specific gene expression. PCR amplification was performed using the following program: 95°C initial denaturation for 10 min, 36 cycles consisting of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, followed by a 5 min final extension at 72°C. Amplified fragments were resolved on 2% agarose gels followed by staining with ethidium bromide. PCR products were isolated from an agarose gel using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and directly sequenced from both directions using the Dye terminator sequencing core kit (PE Applied Biosystems, Warrington, UK). Sequencing was performed on an ABI3700 automated sequencer.

The expression of *USP29* in A9 hybrids and human glioma cell lines was detected using the primers: USP29-2F (5′-GAAATGCAAATTCTTGGGTCA-3′) and USP29-2R (5′-TCGTTTATATAACATAAGCAATCCA-3′). The PCR conditions were as follows: 38 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s.

2.7. *EST clones, 5′-rapid amplification of cDNA ends (RACE) and sequencing*

EST clones AW197312 and H80201 were obtained from the IMAGE Consortium and fully sequenced using the standard (M13) sequencing primers as described above. EST H80201 and EST H78825 corresponded to the cDNA clone (IMAGE: 230170).

To further extend the 5′-end of EST H80201, we performed RACE with a human pituitary gland cDNA template (human pituitary gland Marathon-Ready cDNA; Clontech) by two rounds of RT-PCR with two sets of oligonucleotide primers using *LaTaq* (TaKaRa, Kyoto, Japan). The 5′-modified cDNA was used as template for PCR where 40 cycles of reaction (98°C for 30 s, 54°C for 30 s, 68°C for 4 min) were carried out with final extension at 72°C for 10 min using a primer to adapter sequence (AP1, 5′-CCATCCTAATACGACTCACTATAGGGC-3′) and an EST H78825 specific primer (16-3R, 5′-CAAGGGGAGCAGTCAGTTTC-3′). A thirty-fold dilution of the PCR reaction was used as a template in a second PCR where 40 cycles of reaction (98°C for 30 s, 57°C for 30 s, 68°C for 4 min) were carried out with final extension at 72°C for 10 min using an adapter sequence-specific nested primer (AP2, 5′-ACTCACTATAGGGCTCGAGCGGC-3′) and an EST H80201-specific nested primer 16-2R. To confirm amplification of the PCR products, PCR samples were resolved by electrophoresis through a 2% agarose gel, and then the bands were excised and purified with the Qiagen gel purification kit (QIAGEN). The purified PCR products were subcloned using the pGEM-T Easy Vector System (Promega), and plasmid DNA was isolated with the Plasmid Miniprep kit (MILLIPORE, Bedford, MA, USA). Clones containing the appropriate inserts were sequenced using the standard sequence primers (M13) as described above.

Sequence alignments and database search were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.8. *Methylation-specific PCR (MSP) assay*

Genomic DNAs were treated with sodium bisulfite using the CpGenome DNA Modification Kit (INTERGEN, Purchase, NY, USA) according to the manufacturer's instructions. Primers were designed in CpG island D and E to sites containing frequent cytosines, to distinguish methylated from unmethylated DNA. In this examination, we used two sets of primers (U and M) designed for

Table 1. Summary of the primers sets and PCR conditions.

Primer	Sequence, (5' to 3')	Annealing (°C)	Size (bp)	Genomic position *
P3-D-MSP-U-F	TTGTTGTATTTGTTGTTAATTAATTT	44	123	161790
P3-D-MSP-U-R	TCGTCGTATTTGTCGTTAATTAATTC			161912
P3-D-MSP-M-F	CAAACACTATCCTAATTAATTAAACA	51	123	161790
P3-D-MSP-M-R	GCAAACGCTATCCTAATTAATTAAACG			161912

*Genomic position is the the location of the 5′ nucleotide of the primer in relation to the first base of the sequence in GenBank accession no. AC006115.

annealing to bisulfite-modified genomic DNAs, as summarized in Table 1. One primer set (U) annealed to unmethylated DNA that had undergone chemical modification. A second set (M) annealed to methylated DNA that had undergone a chemical modification. PCR reactions were processed at 35 cycles consisting of 1 min at 95°C, 1 min at the annealing temperature listed in Table 1 and 1 min at 72°C.

2.9. Bisulfite-treated genomic DNA sequencing

Extracted genomic DNA was treated with sodium bisulfite as described above. PCR was performed on modified DNA in which cytosine was converted to uracil using the primers P3-E-Seq-L (5′-TTATTTTTGTTTTAAAAAGG-3′) and P3-E-Seq-R (5′-AATATCCACCCTAAACTAAT-3′) for 40 cycles under the following conditions: 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s. Amplified PCR products were separated and isolated using a 2% agarose gel using the Qiaquick gel extraction kit (QIAGEN). The purified DNA was then cloned into the pGEM-T vector and sequenced from both directions as described above. For each DNA sample, 8–10 clones were analyzed. The number of clones reported varied according to the number of clones with correct inserts and those for which the sequences were complete. We examined a total 33 CpG sites in CpG island E.

3. Results

3.1. Screening for differentially expressed transcripts using monochromosomal hybrids

Prior to EST screening for differentially expressed transcripts, we analyzed the expression profiles of human *PEG3* and *USP29*, that have been known as imprinted genes, in mouse A9 hybrids containing a paternal or maternal human chromosome 19. PCR amplification products for both genes were detected in mouse A9 hybrids with a paternal allele, A9(19P) clones, but not with a maternal allele [A9(19M) clones (Fig. 1A, *USP29* RT+)] as the same pattern of *PEG3* expression in A9 hybrids as previously reported.[8] Neither deletion nor loss of the introduced chromosome was observed in control amplifications of genomic DNA (Fig. 2A, *USP29* DNA).

To determine the allelic expression profiles of a total of 220 ESTs in the chromosome 19q region, RT-PCR analysis was performed using mouse A9 hybrids. Here, we describe the expression profiles of 9 ESTs located on the *PEG3* locus (Fig. 1A). ESTs, H80201, H78825 and AW197312, were expressed only from the paternal chromosome (Fig. 1A, upper four panels, data not shown for H78825). AW592122, AA383002, AA065247 and AI338601, which corresponded to *PEG3*, were also expressed only from the paternal allele, as expected (data not shown). AA833724 was expressed equally from both alleles (data not shown). T52900 expression was not detected in mouse A9 hybrids.

3.2. Imprint-dependent expression of EST H80201 in the PEG3 *locus*

In order to investigate whether the EST H80201 transcripts have an imprinted expression profile, we analyzed cultured lymphoblasts from normal donors and detected exclusive paternal expression in informative cases. Through direct sequencing of PCR products from genomic DNAs of 25 individuals, an A/C polymorphism was identified in the EST H80201 transcribed region. This single nucleotide polymorphism (SNP) corresponded to nucleotide position 164,998 of BAC 470f8 (GenBank Accession No. AC006115) (Fig. 3). Subsequently, we performed RT-PCR on RNA from four informative individuals heterozygous for the A/C polymorphism. Because a diagnostic restriction enzyme site was not available, monoallelic expression was confirmed by direct sequencing of the PCR products (Fig. 4).

Expression of EST H80201 in normal human adult brain, placenta, ovary, spleen and testis was detected by RT-PCR (data not shown). To determine whether expression of H80201 in the brain was imprint-dependent, the above-mentioned SNP was used for expression analysis. Five out of five non-tumor brain tissues of glioma patients showed monoallelic expression, which could be determined because informative heterozygosity was exhibited in genomic DNA (data not shown).

3.3. RACE and sequencing

The EST clones AW197312 and H78825 were completely sequenced. The splice junctions were determined

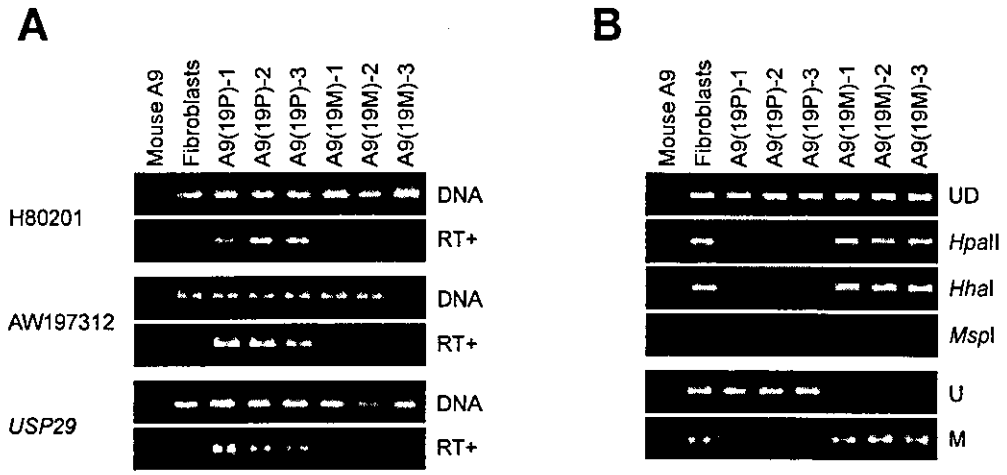

Figure 1. Expression and methylation analysis using A9 hybrids containing a single human chromosome 19. (A) Analysis of parent-of-origin-specific expression of human *PEG3*, *USP29* and ESTs in A9 hybrids. Expression of *PEG3* in mouse A9 cells, human fibroblasts and three mouse A9 hybrids clones each, containing a paternal allele A9(19P) or maternal allele A9(19M), were detected by RT-PCR (RT+). The cDNAs from human chromosome donor fibroblasts, A9 hybrids were amplified using primers for 9 ESTs localized to the human PEG locus. Paternal expression of human ESTs identified within the approximately 8-kb region upstream of *PEG3* exon 1. RT-PCR was performed using primers corresponding to these ESTs. Amplification of genomic DNA using the same primers demonstrated the presence of *PEG3*, *USP29* and ESTs in mouse A9 hybrids (each upper panel DNA). Synthesis of first strand cDNA was verified by PCR of *Gapdh* (data not shown). No RT-PCR products were detected in reverse transcriptase (−) controls (data not shown). (B) Analyses of the methylation status of CpG island D by a methylation-sensitive PCR assay and MSP assay. Genomic DNAs were digested with the methylation-sensitive endonuclease *Hpa* II or *Hha* I prior to PCR amplification. The undigested (UD) and *Msp* I-digested genomic DNAs were amplified using the same primers as the control. In the lower two panels, U indicates the PCR products derived from unmethylated alleles and M indicates the PCR products derived from methylated alleles. Primer sequences for the MSP assay are described in Table 1.

by comparing the EST AW197312 sequence with the genomic sequence of the region (GenBank Accession No. AC006115). The directions of these consensus splice sequences (GT and AG) suggest that EST AW197312 is located directly adjacent to *PEG3*. Furthermore, genomic sequence-matching revealed that the EST AW197312 sequence was located about 200 bp upstream from the first exon of *PEG3*. The RT-PCR analysis using cDNA from human brain indicated that the 5′ end of EST AW197312 was not transcribed with the first exon of *PEG3* (data not shown). We then performed 5′ (RACE) using the Marathon cDNA amplification kit, but did not extend the sequence further forward *PEG3*. 3′ RACE experiments were performed, but no specific RACE amplification products could be obtained. These data suggest that an unidentified gene with a transcription start site located very close to that of the *PEG3* transcription unit exists, and that the transcriptional direction of the EST AW197312 sequence was opposite to that of *PEG3* in a head-to-head orientation (Fig. 4, *ITUP1* variant 2).

The 5′ end of EST H78825 was located within approximately 1.3 kb of the 5′ end of *PEG3*. We performed both 5′ and 3′ RACE, and a 1072-bp 5′ RACE amplification product was obtained and sequenced (DDBJ Accession Nos. AB106535 and AB106536). It confirmed that one of the H78825 exons (putative exon 1) lies approximately 500 bp upstream of *PEG3* overlapping the 5′ AW197312 exon. These data suggest that we had reached one end of the cDNA. However, repeated attempts using 3′ RACE did not succeed in linking downstream sequences. To define the 5′ boundaries of both *PEG3* and the novel imprinted transcript, we carried out RT-PCR using primers spanning the region of *PEG3* exon 1 and the putative exon 1 of the novel imprinted transcript, but no PCR products were detected (data not shown). These data indicate that the novel imprinted transcript was transcribed away from *PEG3* and that its 5′ exon did not overlap with *PEG3* but started approximately 500 bp upstream (Fig. 3, *ITUP1* variant 1). We called these new transcripts *ITUP1* (imprinted transcript 1 oppositely located upstream of *PEG3*).

The EST H78825 probe detected a single, 9-kb transcript in human Northern blots,[9] which was expressed at high levels only in ovary, suggesting that these consensus cDNA sequences may not be complete. Furthermore, analysis of sequences from *ITUP1* variants 1 and 2 did not identify open reading frames encoding a protein.

3.4. *Maternal methylation at the CpG islands in the* PEG3 *locus in mouse A9 hybrids*

We searched for parent-of-origin-specific methylated CpG islands in the *PEG3* locus in mouse A9 hybrids

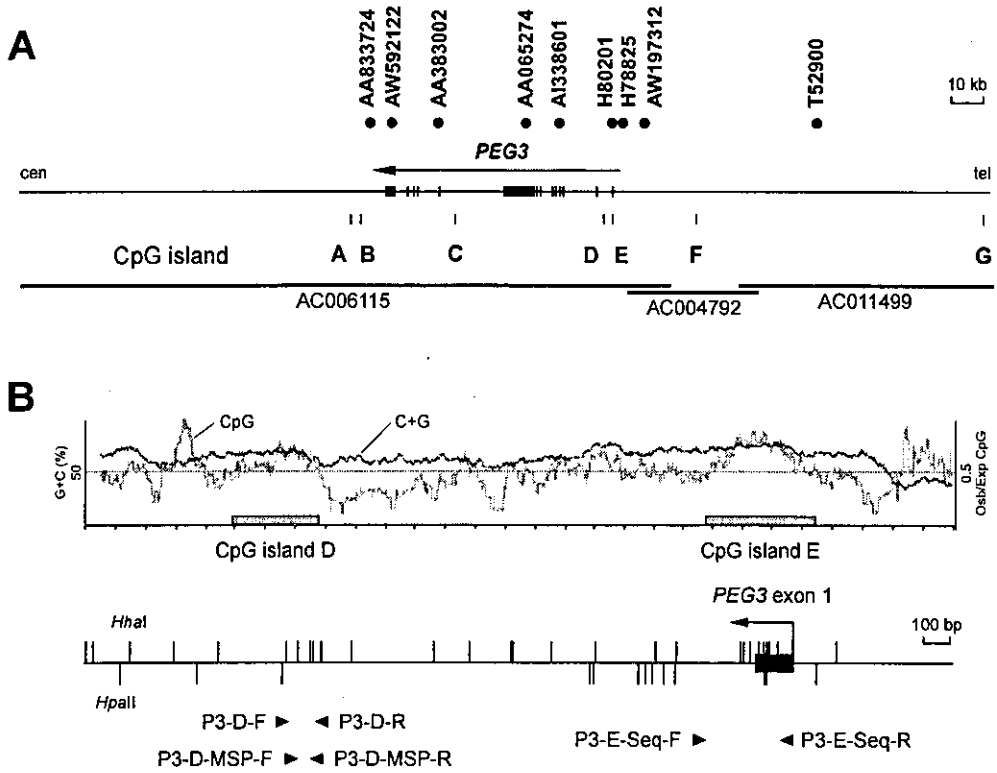

Figure 2. A physical map of the *PEG3* locus. (A) Genomic structure of the human *PEG3* gene at 19q13.4. Exons are indicated by black boxes. The transcriptional direction of *PEG3* gene is indicated with an arrow. CpG islands were identified using the GRAIL program. The genomic contigs are shown by the horizontal bars with accession numbers listed below. ESTs mapped in this region are indicated by dots in relative spacing. (B) The upper panel shows the GC content and CpG observed/expected ratios calculated using CpG view v. 1.5 (Division of Genetic Resources, National Institute of Health, Tokyo, Japan). CpG islands are indicated by gray boxes. In the lower panel, exon 1, which is the transcriptional start site of *PEG3* (arrow) is indicated by a black box. Restriction sites for *Hha* I (upper) and *Hpa* II (lower) are depicted by vertical lines. PCR primers to investigate the status of DNA methylation are indicated by arrowheads with names.

by using a methylation-sensitive PCR assay, and identified a maternal-specific methylated CpG island D as with the CpG island E analysis (Fig. 2B) as reported in Maegawa et al.[8] PCR products for this CpG island were detected only in hybrids containing a maternal allele (Fig. 1B, upper four panels). However, for the other 5 CpG islands, except CpG islands D and E, there were no differences in the methylation pattern between both hybrids containing paternal or maternal chromosome 19. We observed bi-allelic methylation of CpG islands C, F and G (data not shown). Subsequently, the methylation status of CpG island D was confirmed by an MSP assay. Consistent with the results of the methylation-sensitive PCR assay, amplification of sodium bisulfite-treated genomic DNA with primers for CpG island D that should specifically anneal to unmethylated (U) or methylated (M) alleles, confirmed maternal allele-specific methylation in mouse A9 hybrids (Fig. 1B, bottom two panels). The same pattern was observed for CpG island E, as shown previously by Maegawa et al.[8] In addition, almost completely methylated CpGs of CpG island E were detected specifically in the hybrid with a maternal chromosome 19 by bisulfite sequencing analysis [Figs. 5 and 6, A9(19P)-1 and A9(19M)-1].

3.5. *Analysis of methylation at the* PEG3 *locus in human adult brain tissues*

MSP was applied to assess the methylation status of CpG islands D and E in non-tumor brain tissues. Differential methylation was observed in non-tumor brain tissues (data not shown). Subsequently, to confirm the methylation status of CpG island E, we performed bisulfite sequencing analysis. Consistent with the result of MSP, unmethylated or methylated alleles were detected (Figs. 5 and 6, non-tumor brain I and II) although DNA polymorphisms to distinguish the parental alleles were not found in the region we examined.

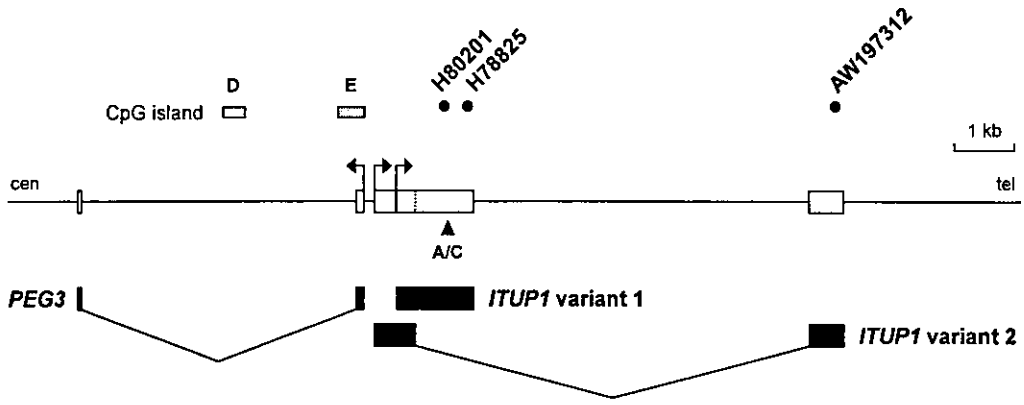

Figure 3. Organization of the *ITUP1* and *PEG3* loci. Exons of these two genes are represented by white boxes. The transcriptional direction of each gene is indicated by arrows. The gray boxes indicate the position of the CpG islands. Dots indicate the ESTs which correspond to *ITUP1* variants 1 and 2. The arrowhead indicates the position of the A/C polymorphic site.

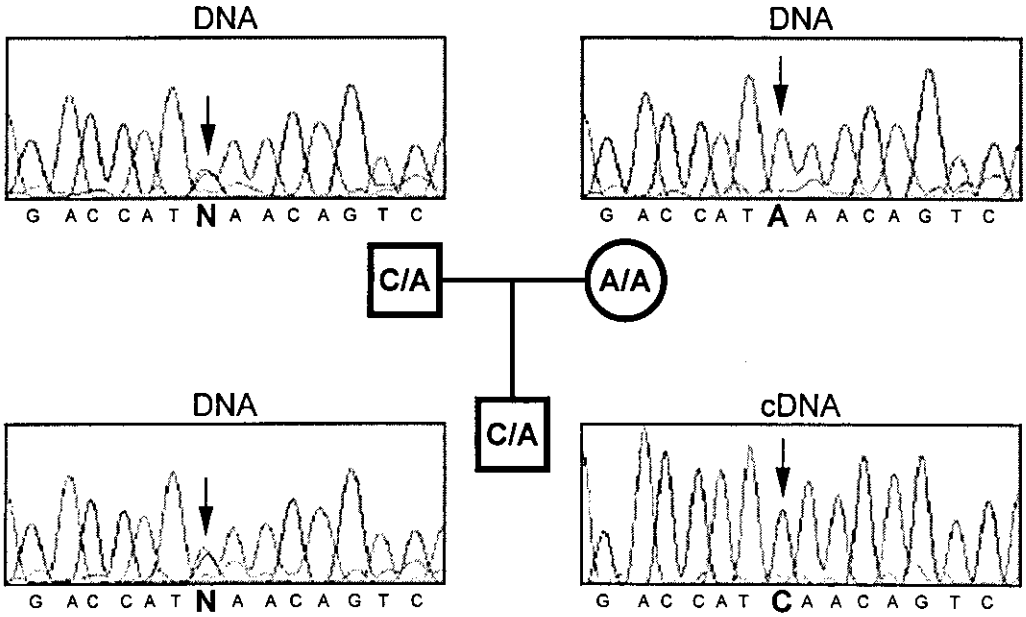

Figure 4. Verification of paternal expression of EST H80201 in normal human lymphoblasts. The allelic expression was assessed by direct sequence analysis. Schematic pedigree of lymphoblast samples. Maternal lymphoblast genomic DNA was homologous for the A allele (upper right DNA panel) for this informative (A/C) lymphoblast sample (bottom left DNA panel), which showed that the C allele was contributed paternally. The cDNA from lymphoblasts (bottom right cDNA panel) was derived from the C allele only (arrow marks the absence of the A allele), demonstrating that the EST was paternally expressed.

3.6. *Correlation of epigenetic silencing of* ITUP1 *and* PEG3 *expression in glioma cell lines depends on DNA methylation of CpG islands*

We analyzed the expression of *ITUP1*, *PEG3* and *USP29* by RT-PCR in glioma cell lines (Table 2). The *PEG3* expression patterns were previously reported.[8] We found that *ITUP1* (variants 1 and 2) and *PEG3* expression were simultaneously downregulated in four glioma cell lines (HTB-16, HTB-17, HTB-138 and CRL-1620), despite strong expression in non-tumor brain tissues (Table 2, data not shown for brain tissues). In contrast, *USP29* was upregulated in one glioma cell line, CRL-2020 (Table 2).

Subsequently, we examined the methylation pattern of CpG island D by MSP in glioma cell lines and obtained similar results with that of CpG island E. Although differential methylation was observed in five *ITUP1*- and *PEG3*-expressing glioma cell lines (HTB-12, HTB-

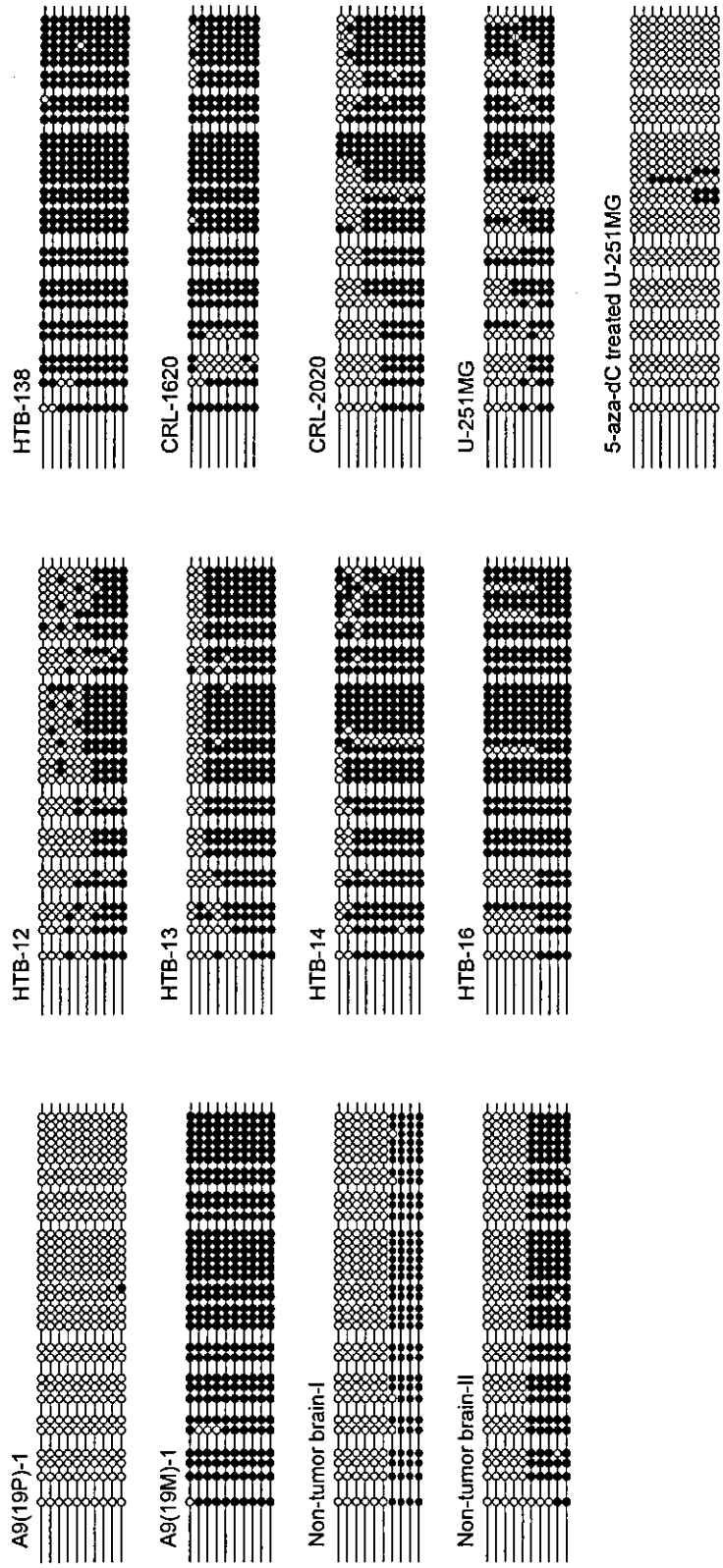

Figure 5. Methylation status of CpG island E in A9 hybrids, non-tumor adult human brain tissues and glioma cell lines. DNA methylation was analyzed by the bisulfite sequencing method. Bisulfite-treated DNA from each tissue and cell lines was amplified with degenerate primers for the CpG island E, and then sequenced as described in Materials and Methods. Individual lines indicate clones sequenced. Circles indicate CpG sites within the regions analyzed. Filled circles and open circles indicate methylated cytosines and unmethylated cytosines, respectively.

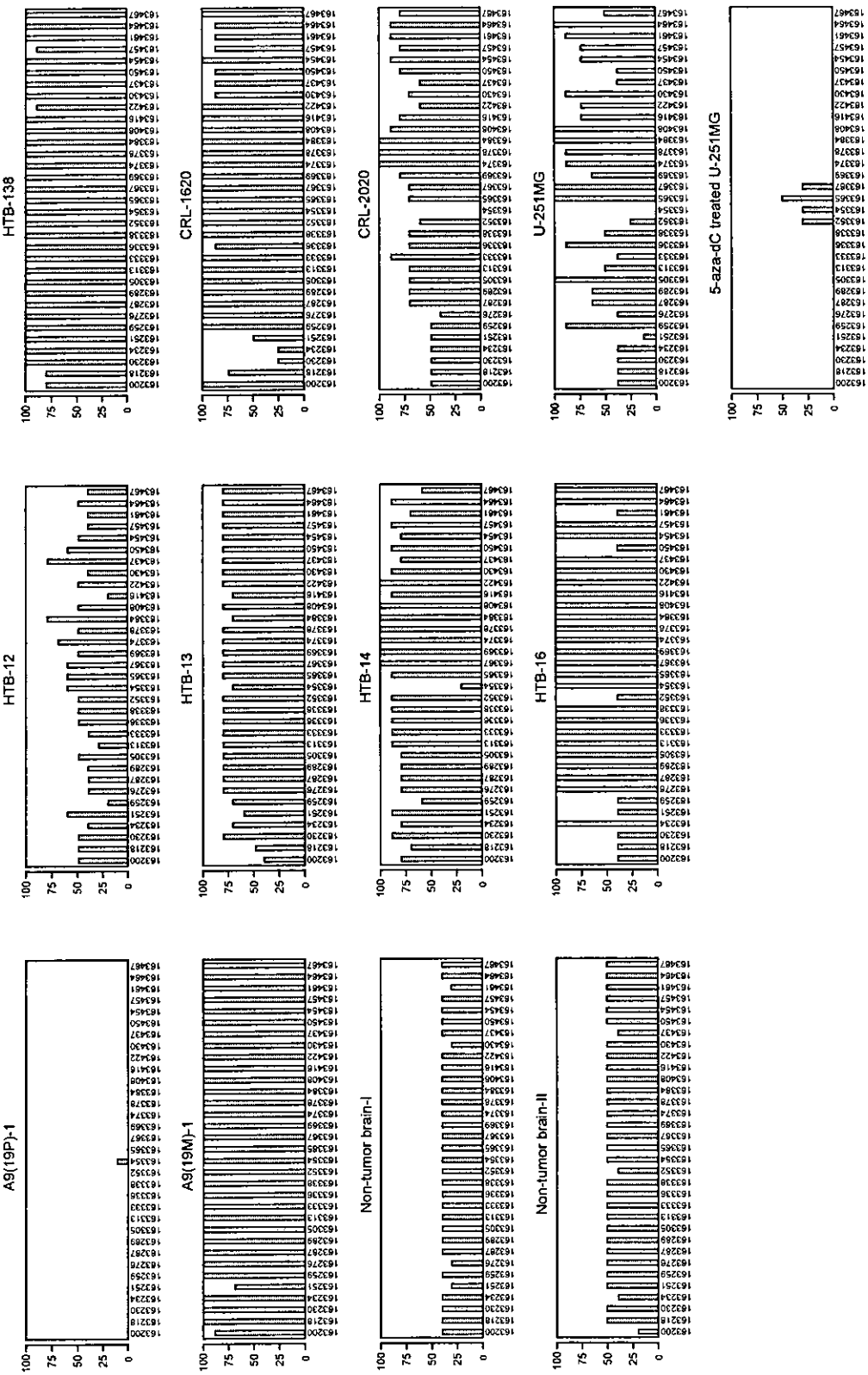

Figure 6. Methylation profiles of the CpG island E in A9 hybrids, non-tumor adult human brain tissues and glioma cell lines. A total of 33 sites in the CpG island E were analyzed by the bisulfite sequencing assay. The percentages of DNA methylation at each CpG site are represented as the lengths of gray vertical lines at the relative positions of the CpG dinucleotide in the genomic sequence (GenBank Accession No. AC006115).

Table 2. Summary of the expression analysis of imprinted genes.

Cell lines	Genes	Genotype	Expressed allele(s) Non treated	TSA-treated	5-aza-dC-treated	5-aza-dC/TSA-treated
HTB-12	*ITUP1*	A/A	+(A)	+(A)	+(A)	+(A)
HTB-13	*ITUP1*	A/C	+(C)	+(C)	+(A/C)	+(A/C)
HTB-14	*ITUP1*	A/A	+(A)	+(A)	+(A)	+(A)
HTB-16	*ITUP1*	A/A	-	-	+(A)	+(A)
HTB-17	*ITUP1*	A/A	-	-	+(A)	+(A)
HTB-138	*ITUP1*	A/A	-	-	+(A)	+(A)
CRL-1620	*ITUP1*	A/A	-	-	+(A)	+(A)
CRL-2020	*ITUP1*	A/C	+(C)	+(C)	+(A/C)	+(A/C)
U-251MG	*ITUP1*	A/A	+(A)	+(A)	+(A)	+(A)
HTB-12	*PEG3*	C/G	+(G)	+(G)	+(C/G)	+(C/G)
HTB-13	*PEG3*	C/G	+(G)	+(G)	+(C/G)	+(C/G)
HTB-14	*PEG3*	C/G	+(G)	+(G)	+(C/G)	+(C/G)
HTB-16	*PEG3*	C/G	-	-	+(C/G)	+(C/G)
HTB-17	*PEG3*	C/G	-	-	+(C/G)	+(C/G)
HTB-138	*PEG3*	C/G	-	-	+(C/G)	+(C/G)
CRL-1620	*PEG3*	C/G	-	-	+(C/G)	+(C/G)
CRL-2020	*PEG3*	C/G	+(G)	+(G)	+(C/G)	+(C/G)
U-251MG	*PEG3*	C/G	+(G)	+(G)	+(C/G)	+(C/G)
HTB-12	*USP29*	ND	-	-	+	+
HTB-13	*USP29*	ND	-	-	+	+
HTB-14	*USP29*	ND	-	-	+	+
HTB-16	*USP29*	ND	-	-	+	+
HTB-17	*USP29*	ND	-	-	+	+
HTB-138	*USP29*	ND	-	-	+	+
CRL-1620	*USP29*	ND	-	-	+	+
CRL-2020	*USP29*	ND	+	+	+	+
U-251MG	*USP29*	ND	-	-	+	+

ND: not detected +: expressed −: not expressed.

13, HTB-14, CRL-2020 and U-251MG), some unmethylated bands were faint (data not shown). Therefore we investigated the methylation status of CpG island E by the bisulfite sequencing method to obtain further information about the methylation status of the CpG island in eight glioma cell lines (HTB-12, HTB-13, HTB-14, HTB-16, HTB-138, CRL-1620, CRL-2020 and U-251MG). Three glioma cell lines (HTB-16, HTB-138 and CRL-1620) that did not express *ITUP1* and *PEG3* exhibited highly methylated alleles (Fig. 5, HTB-16, HTB-138 and CRL-1620). The methylation of CpG island E in the glioma cell lines that maintained the imprint-dependent expression of these two transcripts exhibited an aberrant methylation pattern compared with that of non-tumor adult brain tissues (Fig. 5, HTB-12, HTB-13, HTB-14, CRL-2020 and U-251MG).

We treated glioma cell lines with an inhibitor of cytosine methyltransferase, 5-aza-dC, and/or an inhibitor of histone deacetylase, TSA, and analyzed the expression of *ITUP1*, *PEG3* and *USP29* by RT-PCR. Treatment of glioma cell lines with 100 ng/ml TSA for 24 h did not alter the expression of *ITUP1*, *PEG3* and *USP29* (data not shown). In contrast, treatment of glioma cell lines with 1 μM of 5-aza-dC for 5 days led to bi-allelic expression of *PEG3* and also activated *ITUP1* and *USP29* expression (Table 2). Here we show the data for the glioma cell lines that did not express *ITUP1*, *PEG3* and *USP29*, CRL-1620, and the monoallelic *PEG3*-expressing cell line, U-251MG. Because none of the four *ITUP1*-expressing glioma cell lines (HTB-16, HTB-17, HTB-18 and CRL-1620) were informative, the allelic expression of *ITUP1* could not be determined. The same results were obtained from other glioma cell lines (HTB-12, HTB-14, HTB-17 and HTB-138) which we examined (data not shown).

MSP was applied to assess the methylation status of CpG island D in 5-aza-dC-treated glioma cell lines. Only unmethylated bands were observed (data not shown), as with the CpG island E analysis. Demethylation of CpG island D was confirmed in a 5-aza-dC-treated glioma cell line, U-251MG, by using the bisulfite sequencing method (Figs. 5 and 6, 5-aza-dC-treated U-251MG). These results show that differential methylation of this CpG island has an important role in the maintenance of monoallelic expression of *PEG3* and possibly of *ITUP1*, and that silencing of these two transcripts in glioma cell lines is caused by aberrant methylation.

4. Discussion

4.1. Identification of a novel imprinted transcript on human chromosome 19

By screening using these mouse A9 hybrids, we identified three paternally expressed ESTs and two mater-

nal allele-specific methylated CpG islands. A novel imprinted transcript, *ITUP1*, located at the homologous region of the first non-coding exon of mouse *Usp29*, in a head-to-head orientation to *PEG3*, was expressed from the paternal genome in lymphoblasts and possibly in the adult brain. Furthermore, differential methylation was also observed in non-tumor brain tissues. The imprinted status was faithfully maintained in mouse A9 hybrids, as was the case for other chromosomes.[13–16] Therefore, this assay system has provided a valuable resource for the efficient identification of imprinted genes in humans and for the investigation of the epigenetic mechanisms of genomic imprinting.

4.2. Expression pattern of ITUP1 *and other imprinted genes on* PEG3 *locus*

We performed RT-PCR analysis and observed the expression of EST H80201, corresponding to *ITUP1*, in adult human tissues; brain, placenta, ovary, spleen and testis, and fetal human tissues; brain, kidney, thymus, liver (data not shown). In addition, low levels of EST H80201 expression was detected in lymphoblasts. However, human *USP29* was not transcribed at significant levels in brain when assayed by RT-PCR analysis (data not shown). Furthermore, *USP29* did not show a similar expression profile to *ITUP1* in glioma cell lines. Although we did not determine whether *USP29* had a monoallelic expression profile, we discovered that *USP29* was up-regulated in one glioma cell line, CRL-2020. Thus, the *ITUP1* sequence comprises the first non-coding exon of *Usp29*, but was not co-transcribed with *USP29* in human brain. Kim et al. reported Northern blot analysis of the ESTs matching this sequence located at the 5′-end of *PEG3*. Using a probe adjacent to the 5′ end of *PEG3*, they detected a single 9-kb transcript which was highly expressed only in the ovary. A probe complementary to the coding exon of *USP29* enabled detection of a 3.5-kb transcript exclusively expressed at low levels in the adult testis.[9] Our data also suggest that these two transcripts of cDNA sequences might represent different transcription units within the human genome.

We observed the bi-allelic expression of *PEG3* and activation of *ITUP1* and *USP29* expression in glioma cell lines treated with an inhibitor of cytosine methyltransferase, 5-aza-dC. The activation of these three imprinted genes was not observed in glioma cell lines following TSA treatment. Although the reason for this silencing remains to be clarified, complexes containing methyl-binding proteins but not histone deacetylase may be involved in the silencing of *ITUP1*, *PEG3* and *USP29*. Our data indicates that *USP29* and *PEG3* transcripts are regulated by different genetic elements, at least in human brain, and that they most likely use different promoters as well. The imprinting status of these three transcripts appeared to be regulated by the methylation of their respective DMRs rather than by histone acetylation.

The 5′-ends of the *Peg3* and *Usp29* genes are located less than 150 bp apart in the mouse. It therefore seems likely that the two genes share regulatory elements that control transcriptional sites and transcriptional levels of each gene, and possibly allele-specific expression. In fact, the expression patterns of mouse *Usp29* and *Peg3* are very similar: both genes are expressed in embryos and at significant levels only in adult brain.[9] Szeto et al. reported an additional imprinted gene in mouse, ossification center-associated transcript (*Ocat*), on this *Peg3* locus.[19] *Ocat* showed a dissimilar expression profile to *Peg3* in embryos, suggesting that they are regulated by independent genetic elements. However, *Ocat* and *Peg3* are both paternally expressed genes, and it is possible that their imprinted expression is under the same control. How whether these imprinted genes on this *Peg3* locus are coordinately or independently regulated remains to be discovered. It may be possible that the mechanisms for regulation of these genes may vary. Further investigation is needed to explain these differences in gene expression.

As for the regulatory mechanisms for the newly identified paternally expressed *ITUP1* and *PEG3*, the physical distance between the transcription start sites of the *ITUP1* and *PEG3* genes is approximately 200 bp, suggesting that regulation of the expression of both genes may be coordinated though a bidirectional promoter. In fact, using the polymorphic site which was identified, we showed that *ITUP1* and *PEG3* were expressed monoallelically in non-tumor human brain tissues. All non-tumor brain tissues we examined had a combination of unmethylated and methylated alleles, suggesting that monoallelic expression of *ITUP1* and *PEG3* was regulated by methylation of CpG islands D and E. Furthermore, these genes showed similar expression profiles in glioma cell lines, suggesting that they are regulated by the same genetic elements. These observations support the idea that the transcription and imprinting of *ITUP1* and *PEG3* might be jointly controlled through the methylation of a shared genomic region, at least in human brain.

4.3. ITUP1 *and* PEG3 *repression related to DNA methylation in glioma cell lines*

At least 20 loci in which two genes are located in a head-to-head orientation have been reported.[20] Recently, 290 genes with housekeeping functions were examined in order to find additional loci with such bidirectional structures, and 23% (66 genes) exhibited divergent bidirectional transcription units within 300 bp of each other. Most of the genes exhibiting this arrangement were implicated in DNA repair and the citric acid cycle. A common feature of these bidirectional loci is the presence of a CpG island between the genes. One possibility is that

two genes might share one CpG island for purposes of coordinate expression. Though in many cases the two genes did not have an obvious functional relationship, some of these genes were coordinately regulated by bidirectional activity of a CpG island promoter. Several tumor suppressor genes are organized in a head-to-head structure. For example, The NBR2 gene is located head-to-head with *BRCA1*.[21]

In our current study, we observed similar expression profiles of *ITUP1* and *PEG3* in glioma cell lines. The methylation status of CpG island E was altered in all tested glioma cell lines compared with that of non-tumor brain tissues. Hypermethylated alleles were observed in glioma cell lines HTB-16, HTB-138 and CRL-1620 that silenced *ITUP1* and *PEG3* expression. It is possible that the methylation status of DMR is altered by hypermethylation in glioma carcinogenesis, because hemimethylated CpG sites were found in 8 eight out of 33 CpG sites in glioma cell line HTB-16. We found some unmethylated alleles (not heavily methylated) in glioma cell lines HTB-12, HTB-13, HTB-14, CRL-2020 and U-251MG that maintained allele-specific expression. The methylation status of CpG island E exhibited aberrant patterns compared with that of non-tumor brain tissues. Matching the expression profiles and DNA methylation pattern of CpG island E suggests that differential methylation is critical for allelic expression pattern of *ITUP1* and *PEG3*. The hypermethylation leads to silencing of these two genes in glioma cell lines. Although it is not clear how often this epigenetic silencing appears in gliomas, our results show that the inactivation of *ITUP1* and *PEG3* by aberrant cytosine methylation in glioma cell lines occurs with a frequency of about 40%. The frequencies of methylation-associated gene inactivation in various cancer cells is relatively low, at around 20%.[22–27] Thus, this percentage might be influenced by the accumulation of cytogenetic alterations that have conferred a selective growth advantage. The analysis of the methylation status of CpG island E in glioma specimens may explain this phenomenon. Furthermore, recent studies have shown that there are conserved Gli-type transcription factor YY1-binding motifs located in the first intron of mouse and human *PEG3*, and the binding to this motif is methylation-sensitive and that all of the YY1-binding sites are differentially methylated between two parental alleles *in vivo*. The functional assays of this region suggest that the YY1-binding region acts as a methylation-sensitive insulator that may play a role in the imprinting control of this domain in mice.[28] Therefore, analysis of the methylation status of YY1-binding sites in CpG island D is also important to define the mechanism of silencing of *PEG3* expression in glioma cell lines.

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