mour. In a case-cohort study of 78 patients with favourable histology Wilms' tumour, the research group compared tumour telomerase levels in patients with and without eventual recurrence, measuring TERT activity, TERT mRNA and expression of the RNA component. In their study 81% had detectable telomerase activity, 97% had detectable TERT transcriptand 100% had detectable hTR. Of the variables assessed, only TERT mRNA expression correlated with tumour recurrence marker [127]. Recently the same group studied a larger set of 244 Wilms' tumours and found that high telomerase RNA expression level is an adverse prognostic factor for favourable-histology Wilms' tumour [152]. Although these reports may reflect the level of telomerase expression in Wilms' tumour, the link between high TERT expression and unfavourable prognosis (whether it is causative or correlative) has not been addressed experimentally. An alternative focus for research is whether telomere dysfunction, rather than high telomerase level, correlates with high-risk tumours. In a recent study abnormal telomere shortening was found in cultured cells and in tissue sections from highly aggressive Wilms' tumours. Dysfunctional telomeres were associated with specific cell division abnormalities, including anaphase bridges and multipolar mitoses. Telomere-dependent mitotic instability was found to be present in a subgroup of Wilms' tumours, predominantly consisting of high-risk tumours [95].

HEPATOBLASTOMA (HB)

Hepatic tumours comprise approximately 5% of the total neoplasms occurring in the foetus and neonate [153]. HB is one of the common paediatric tumours and more than 70% of the tumours are diagnosed in children less than 2 years old [153, 154]. This tumour, which is derived from hepatic precursor cells, is morphologically similar to immature hepatocytes and the prognosis of the patients is variable [155, 156]. Data on telomere maintenance in childhood HB neoplasms, including TERT gene dosage and mRNA levels, is sparse. Hiyama *et al.* have detected TERT mRNA expression in 96% of 61 primary HB in patients listed in the Japanese Study Group of Pediatric Liver Tumour between 1991 and 2002 [157]. The group showed that an increased level of TERT mRNA expression or telomerase activity is a prognostic indicator of poor outcome in children with HB, independent of disease stage and histological classification. Since telomerase activity has been detected in HB, the use of telomerase inhibitors may provide an attractive approach to therapy. To that end, a few studies using different approaches have succeeded in inhibiting TERT using siRNA and the hTR template antagonist oligonucleotide (GRN163L), tamoxifen, or interleukin-4(hIL-4) gene transfer and have demonstrated a reduction in cell proliferation, an increase in cell differentiation and apoptosis [158-161]. Although it would need a larger series to clarify the correlation between clinical variables and the levels of TERT mRNA or the inhibition of telomerase activity, these findings have drawn attention to the fact that high telomerase activity may stratify patients who are likely to have cancer recurrence requiring postoperative aggressive chemo-adjuvant therapy or, in the future, telomerase-targeting therapy.

THERAPEUTIC STRATEGIES TARGETING TELOMERE MAINTENANCE

The interest in telomere maintenance mechanisms in a cancer therapeutics context has emerged following the observations that: (i) Immortality of cancer cells is intimately related to the maintenance of the ends of human chromosomes [25, 63, 162-164]; (ii) Telomerase activity is detectable in over 85% of human tumour samples *in vivo* and some studies have suggested that cancer stem or stem-like cells are also telomerase-positive [15-18]. The reactivation of telomerase in cancer cells stabilizes telomere length, thereby counteracting the cell division-related telomere erosion and providing unlimited proliferative capacity to malignant cells; (iii) Telomerase repression and tight regulation in humans function as tumour suppressor mechanisms [19]; and (iv) Telomerase is usually not expressed in normal tissues of somatic origin and is expressed only transiently or at low levels in proliferative tissues including hematopoietic progenitor cells, intestinal crypt cells, endometrial cells and basal layer cells of skin and cervical keratinocytes.

The early findings that HeLa cells express telomerase [165] and that immortalization of cells *in vitro* occurs concomitantly with the activation of telomerase activity [64] have established a close link between the limitless replicative potential of cancer cells and the maintenance of the telomeres sequences. The key finding that related the telomeres to cancer emerged later when it was shown that ectopic expression of the TERT subunit in cultured human retinal pigment epithelial cells or foreskin fibroblasts extended their life span and conferred an indefinite proliferative potential to the cells [166], also elongating the short telomeres by 2.5 kbp [167]. This concept was coupled with the remarkable reports by Hahn showing that cloning a mutant TERT gene into a cancer cell *in vitro* causes the cell to lose the ability to form tumours in mice, leads to shortening of telomeres and forces the cell into replicative senescence [162, 168]. These results argue strongly that telomere maintenance is the key to cell immortality and establish telomere length as the clock that keeps track of cell division. Soon afterwards cumulative reports continued to demonstrate and provide evidence for the genetic validation of telomere maintenance as an anticancer target [169-171]. Moreover, the concept of a close relationship between cancer cell survival and telomere/telomerase biology presented novel molecular targets for cancer therapy and accordingly a number of different approaches have been developed to inhibit telomere preservation in human cancer cells. The most advanced of these is inhibition of telomerase.

Different strategies targeting various telomerase regulatory levels have been reported and can be classified as either targeting production of the telomerase complex using antisense, siRNA, dominant negative telomerase complex approaches or targeting telomerase activity with specific inhibitors (Fig. **4A**), such as: oligonucleotide inhibitors that target the template portion of hTR and small-molecule inhibitors including reverse transcriptase inhibitors (Fig. **4B**), nucleotide analogs and other small molecules (Table **2**). One of the recent and more promising areas of research is targeting the telomere with G-quadruplex stabilizers (Fig. **4C**) [19, 172-176]. The main advantage of targeting telomerase is its wide expression in tumour tissues and its specificity for cancer cells, including putative cancer stem cells. In fact no other tumour-associated gene is as widely expressed in cancer. Importantly, the purely transient expression of telomerase in normal tissues and the longer telomeres in normal stem cells compared with those in tumour cells, reduce the probability of toxicity in normal cells, suggesting that telomerase targeting therapy could have a broad therapeutic window. However, the possibility that cancer therapy targeting telomerase might cause undesired toxicity in telomerase-expressing normal proliferating cells remains to be elucidated by the administration of telomerase inhibitors in long-term clinical trials. It is worth noting that recurrent tumours are characterized by immortal cells that have reactivated telomerase [177-179], hence telomerase inhibitors may also be useful when traditional anti-tumour therapies, which are generally more effective against early stage cancer, have failed. Therefore, anti-telomerase strategies promise to be a novel anticancer approach that might also prove effective against disseminated advanced tumours.

Another promising approach for intervention in telomere maintenance is the disruption of the telomere capping structure at the end of the telomeric sequences. Telomere-capping function requires the integrity of shelterin protein complex (that remodels linear telomeric DNA into T-loops), sufficient telomere length and telomerase expression to maintain the equilibrium of telomere length in the case of critical telomere shortening. Loss of the telomere repeats, mutations of the telomere-associated proteins, or telomerase inhibition may lead to the destruction of telomere structure, which

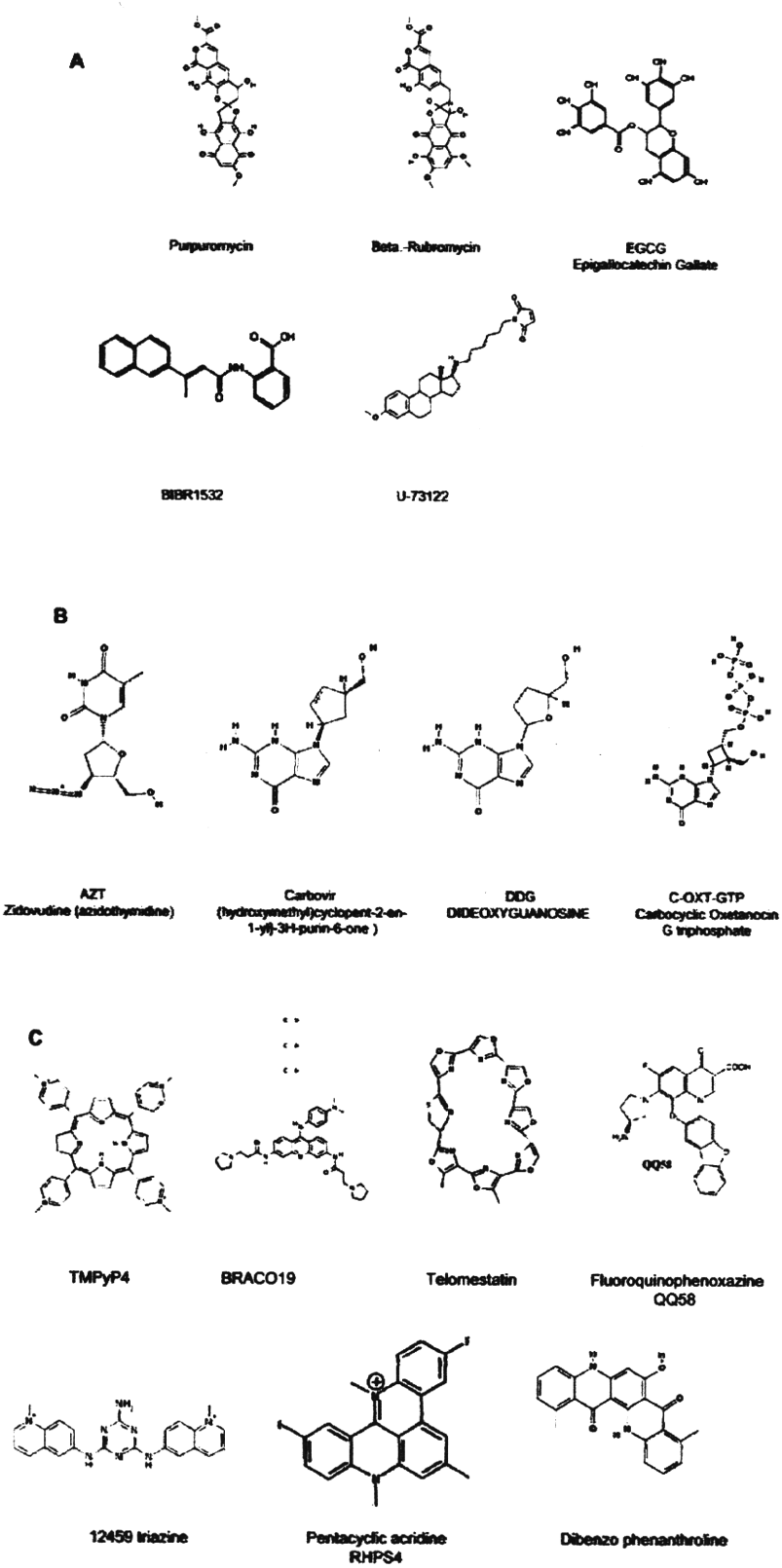

Fig. (4). Telomerase inhibitors.
(A) Small molecules telomerase inhibitors **(B)** Reverse transcriptase inhibitors. **(C)** Small molecules G quadruplex inhibitors

Table 2. Strategies for Targeting Telomere Maintenance

Target	Mode of Action	Inhibitors	References
Telomerase	**Agents blocking telomerase biosynthesis** 1) Oligonucleotide inhibitors : A) Antisense: Prevent translation / promote mRNA degradation • Against hTR • Against hTERT mRNA	Chemically modified oligonucleotides including: phosphorothioates, RNA oligomers nucleotides with alkyl modifications at the 2′ position of the ribose, 2′,5′-oligoadenylate antisense oligomers, N3′→P5′ (GRN163*) phosphoramidates and peptide nucleic acids (PNAs)	[10, 219-225]
	B) Small interfering RNAs, (siRNA): Targeting protein destabilize • Against hTR • Against hTERT mRNA	Chemically synthesized derivatives of natural toxic compounds	[226-228]
	2) Small molecules inhibitors: Targeting the active site of hTERT.	Purpuromycin, BIBR1532, U-73122, Epigallocatechin Gallate, FJ5002	[114, 229-233]
	TERT mutants: Inhibition of the telomerase catalytic protein subunit	Dominant negative TERT constructs mutants that are catalytically inactive.	[234-236]
	Agents blocking reverse transcriptase Blocks chain elongation using the reverse transcriptase enzyme.	Azidothymidine (AZT), carbovir, Dideoxyguanosine (DDG)	[237-240]
	Indirect telomerase inhibitors: Compounds affects telomerase via an indirect mechanism.	Heat shock protein 90 (HSP90), protein kinase C, histone deacetylase, COX-2 and tyrosine kinase inhibitors, tamoxifen as well as nonsteroidal anti-inflammatory drugs and anti-oxidants	[224,241]
	Immunotherapy Using hTERT as a tumour antigen.	GRNVAC1* (Autologous dendritic cells transduced with TERT) GV1001* (TERT peptide p611-626) p540-548* (TERT peptide p540-548) Vx01* (TERT cryptic peptide p572Y-580 and native TERT p572(R)-580) TLI* (TERT fragment-transduced B-lymphocyte immunization)	Reviewed in [9]
	Suicide gene therapy Using telomerase promoter-driven expression of a toxic gene (for example, oncolytic viral replication or toxic prodrug conversion		[242-244]
Telomere maintenance distruption	**G-quadraplex-stabilizing molecules** Inhibiting telomerase and/or telomere maintenance.	2,6 diamidoanthraquinone, TMPyP4 Cationic porphyrin, BRACO19 Trisubstituted acridine, Telomestatin, Dibenzo phenanthroline, Ethidium derivative, 12459 triazine, Pentacyclic acridine RHPS4, Fluoroquinophenoxazine QQ58,	[4, 245]
	Interference with telosome proteins Telomere dysfunctional telomeres	• TRF1 overexpression • Inhibition of TRF2 • Pin2 mutant	[224, 246-250]

* Telomerase inhibitors in clincal trias.

is recognized as DNA damage [11, 180-184]. Identifying shelterin proteins and understanding how they are involved in telomere maintenance has enriched the list of possible targets for therapeutic intervention. Genetic and biochemical studies suggest that targeting components of shelterin, such as TRF2 [185] or POT1 [14, 186, 187], or exposing the telomere 3` overhang [188] can activate a DNA damage checkpoint response and in so doing induce telomere-initiated senescence or apoptosis in cancer cells [189]. Optimal telomerase activity requires the unfolding of the single-stranded substrate that gives access to the telomerase RNA to allow priming and elongation of the telomere length. Therefore, it has been hypothesized that molecules that selectively bind to and stabilize the telomere sequence in unfolded structures such as quadruplexes may interfere with telomere conformation and telomere elongation via telomerase [190].

Quadruplex nucleic acids are defined as higher order four-stranded structures formed by DNA sequences containing at least one contiguous tract of guanine nucleotides. The occurrence of such sequences in human telomeres and within genomic sequences has been recognised for many years. Recently the diverse structures of G-quadruplexes have been the focus of attention as novel anticancer targets since their formation inhibits the telomerase complex from maintaining telomere length in cancer cells [191-193]. The concept of telomeric quadruplex DNA as a therapeutic target was established with the finding that a group of disubstituted amidoanthraquinone small molecules, containing a planar aromatic chromophore, could inhibit telomerase activity [194, 195]. The hypothesis underlying this observation was that a ligand molecule can induce the single-stranded telomeric DNA substrate to fold into a quadruplex structure, which is known to be incompatible with telomerase-catalysed telomere elongation [190]. More recent studies have provided compelling evidence that the ligand effectively competes with hPOT1 and telomerase for the single-stranded overhang [196-199]. The formation of a quadruplex-ligand complex at telomere ends appears to be equivalent to the exposure of damaged DNA, since it elicits a rapid DNA damage response that is lethal to the affected cells [11, 200], reviewed in [191]. Notably, however, it

was observed that G-quadruplex ligands also induce a short-term response of growth arrest and apoptosis before any detectable telomere shortening, a finding which cannot be explained solely by telomerase inhibition but which to a certain extent indicates that the direct target of these ligands is the telomere dysfunction rather than telomerase inhibition [170, 201-203]. Studies which reported that G-quadruplex ligands block the proliferation of ALT cell lines, together with the finding that neither overexpression nor the introduction of dominant negative domain of TERT in a telomerase positive cell line modify the antiproliferative effect of the G-quadruplex ligand, provided further evidence that the antiproliferative effect of G-quadruplex ligands is independent of telomerase inhibition [204-206]. Chromosome end-to-end fusion with typical images of telophase bridges is yet another evidence that supports the proposal that G-quadruplex ligands primarily act by disrupting the telomere structure [207]. Such telomeric dysfunction has been observed in diverse cancer cell lines treated with different quadruplex ligands [203, 208-210]. No specific inhibitor has so far been specifically designed to inhibit the ALT pathway. Telomere interacting agents targeting telomere sequence or telomere structure however could, in principle, act both on telomerase positive and ALT cells [211-213].

CONCLUSION

In summary, the high proliferation ability of embryonal cells during development requires the establishment of a safe telomere maintenance mechanism for counteracting the shortening of chromosomal termini. Dysregulated, unlimited proliferation and the ability to bypass senescence are acquired capabilities of cancerous cells. Within the context of our current knowledge concerning telomere biology in embryonal tumours, telomerase activity has been detected in most advanced embryonal tumours of childhood. The emerging view of a close relationship between embryonic development, telomere/telomerase biology and embryonal cancer encourages new approaches in the development of innovative therapeutic interventions for childhood malignancy. In pre-clinical studies, telomerase inhibitors have shown promise as effective agents for a wide variety of malignancies and for some embryonal tumours such as NB and MB, but their usefulness in clinical practice has yet to be proven and more research is definitely required to define how telomere biology can be utilised to clinical advantage in malignancies of childhood. At the current state of knowledge it is still not possible to decide the best therapeutic target, between telomerase and telomeres. Although telomerase activation is not the sole mechanism responsible for maintaining telomeric sequences, the enzyme appears to be crucial in the maintenance of telomeres in most embryonal cancers and it has been found to be associated with unfavourable outcomes. However, results vary according to tumour type. Small molecules that target G-quadruplexes in telomeric DNA disrupt telomere maintenance in cancer cells and hence become attractive potential anticancer agents. Genetic-based validation studies have provided a compelling argument which suggests that the telomere maintenance pathway is a well validated target at the preclinical level; hence interference with telomere maintenance may provide an attractive approach to therapy for these deadly diseases and may prove most effective in reducing the risk of relapse by targeting cancer stem cells. Although telomerase and telomere maintenance mechanisms may not yet be the universal targets for anti-cancer therapy, we certainly believe that they will be important targets in future research aimed towards a successful strategy for curing childhood cancer.

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CONFLICT OF INTEREST STATEMENTS

No conflicts of interest exist among the authors related to this project.

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Short Report

Paternal mosaicism of an *STXBP1* mutation in OS

Saitsu H, Hoshino H, Kato M, Nishiyama K, Okada I, Yoneda Y, Tsurusaki Y, Doi H, Miyake N, Kubota M, Hayasaka K, Matsumoto N. Paternal mosaicism of an *STXBP1* mutation in OS.
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Ohtahara syndrome (OS) is one of the most severe and earliest forms of epilepsy. We have recently identified that the *de novo* mutations of *STXBP1* are important causes for OS. Here we report a paternal somatic mosaicism of an *STXBP1* mutation. The affected daughter had onset of spasms at 1 month of age, and interictal electroencephalogram showed suppression-burst pattern, leading to the diagnosis of OS. She had a heterozygous c.902+5G>A mutation of *STXBP1*, which affects donor splicing of exon 10, resulting in 138-bp insertion of intron 10 sequences in the transcript. The mutant transcript had a premature stop codon, and was degraded by nonsense-mediated mRNA decay in lymphoblastoid cells derived from the patient. High-resolution melting analysis of clinically unaffected parental DNAs suggested that the father was somatic mosaic for the mutation, which was also suggested by sequencing. Cloning of PCR products amplified with the paternal DNA samples extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutation, respectively. This is a first report of somatic mosaicism of an *STXBP1* mutation, which has implications in genetic counseling of OS.

Conflict of interest

None of the authors has any conflict of interest to disclose.

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Ohtahara syndrome (OS), also known as early infantile epileptic encephalopathy with suppression-burst, is one of the most severe and earliest forms of epilepsy (1). It is characterized by early onset of seizures, typically frequent epileptic spasms, seizure intractability, characteristic suppression-burst patterns on electroencephalogram (EEG), and poor outcome with severe psychomotor retardation (2, 3). Brain malformations such as cerebral dysgenesis or hemimegalencephaly are often associated with OS, but cryptogenic or idiopathic OS is found in a subset of OS patients, in whom genetic aberrations might be involved (4). Mutations in *ARX* gene have been found in several male patients with OS (5–8). We have recently found *de novo* mutations in *STXBP1* (encoding syntaxin binding protein 1, also known as MUNC18-1) in individuals with cryptogenic OS (9). A microdeletion involving *STXBP1* and various kinds of point mutations including missense, frameshift, nonsense, and splicing mutations have been found in about one-third of Japanese cases with cryptogenic OS (10). We have showed that both missense mutations and

a splicing mutation result in haploinsufficiency of *STXBP1*: degradation of STXBP1 proteins with missense mutations and nonsense-mediated mRNA decay (NMD) associated with an aberrantly spliced mRNAs (10).

Here we describe a family with an affected daughter with an *STXBP1* mutation and healthy parents. Parental analysis indicates that the father is somatic mosaic for the mutation. Detailed molecular analysis is presented.

Materials and methods

Patient and her parents

The 1-year-old girl is a product of unrelated healthy parents. There is no history of epilepsy in her parents. She was born at term without asphyxia after an uneventful pregnancy. Her physical and neurological findings were normal until vomitting, which was supposed to be a presymptomatic event of seizures, was observed at 25 days of age, and her seizures started at 37 days of age, consisting of brief tonic spasms, occasionally in cluster, followed by vomiting and subtle seizures, such as head extension, upward eye gazing, and vocalization, with increased muscle tone of her extremities for a few seconds. According to suppression-burst pattern on EEG (Fig. 1a,b), she was diagnosed as OS. Brain magnetic resonance imaging (MRI) showed normal brain structure (Fig. 1c–f). Seizures were refractory to antiepileptic drugs, such as high-dose phenobarbital, phenytoin, zonisamide, pyridoxal phosphate, valproic acid, ketogenic diet, and potassium bromide. Injection of adrenocorticotropic hormone (ACTH) was partially effective. She was hypertonic and could not control her head or smile. At 6 months of age, a mild rigospastic quadriplegia was noted. Developmental milestones were profoundly delayed.

DNA samples

Peripheral blood leukocytes from the patient and her parents as well as other tissues from the father were used for this study. Genomic DNA from whole blood, saliva, buccal cells, and nails were isolated using a Wizard Genomic DNA Purification Kit (Promega, Tokyo, Japan), an Oragene DNA kit (DNA Genotek, Ottawa, Canada), an ISOHAIR kit (Nippon Gene, Toyama, Japan), and a Gentra Puregene Buccal Cell Kit (Gentra, Minneapolis, MN), respectively. Experimental protocols were approved by Institutional Review Boards for Ethical Issues at Yokohama City University School of Medicine and Yamagata University Faculty of Medicine. Informed consent was obtained from the patient's parents in agreement with the requirements of Japanese regulations.

Mutation analysis and TA cloning

Mutation screening of *STXBP1* by high-resolution melting (HRM) analysis using RotorGene-6200 HRM (Corbett Life Science, Brisbane, Australia) was performed as previously described (10). Parentage was confirmed by microsatellite analysis (9). For measurement of the ratio of wild-type and mutant alleles, PCR products using paternal DNA as a template were subcloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Cloned fragments were amplified with PCR mixture containing 1 × ExTaq buffer, 0.2 mM each dNTP, 0.5 μM each primer, and 0.375 U Ex TaqHS polymerase (Takara Bio, Ohtsu, Japan). M13 forward (5′-TAAAACGACGGCCAGTGAAT-3′) and M13 reverse (5′-CAGGAAACAGCTATGACCAT GA-3′) primers were used for amplification, and an ex10-F (5′-AGCTGAAGAGGGTTCGAT GA-3′) primer was used for sequencing.

RNA analysis

RNA analysis using lymphoblastoid cells (LCL) was performed essentially as previously described (10). Briefly, after incubation with dimethyl sulfoxide (as vehicle control) or 30 μM cycloheximide (Sigma, Tokyo, Japan) for 4 h, total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Tokyo, Japan). Two micrograms total RNA was subjected to reverse transcription, and 1 μl cDNA was used for PCR. Primer sequences are ex9-F (5′-CCCTGTGCTCCATGAATTGAC TTT-3′) and ex12-R (5′-CTGAGGCATCTTCTTC AGCATCTGG-3′). Inhibition of NMD was estimated according to the density ratios of lower normal and upper aberrant bands with/without 30-μM cycloheximide treatments in the culture of the patient's LCL. Two separately extracted RNA samples were used for duplicated experiments, respectively. Data were averaged and the standard deviation was calculated. Statistical analyses were performed using the unpaired Student's *t*-test (two-tailed). DNA of each PCR band purified by QIAEXII Gel extraction kit (Qiagen, Tokyo, Japan) was sequenced.

Results

Through the screening for *STXBP1* mutations in individuals with cryptogenic OS, we found a patient harboring heterozygous c.902+5G>A mutation. To examine whether the mutation

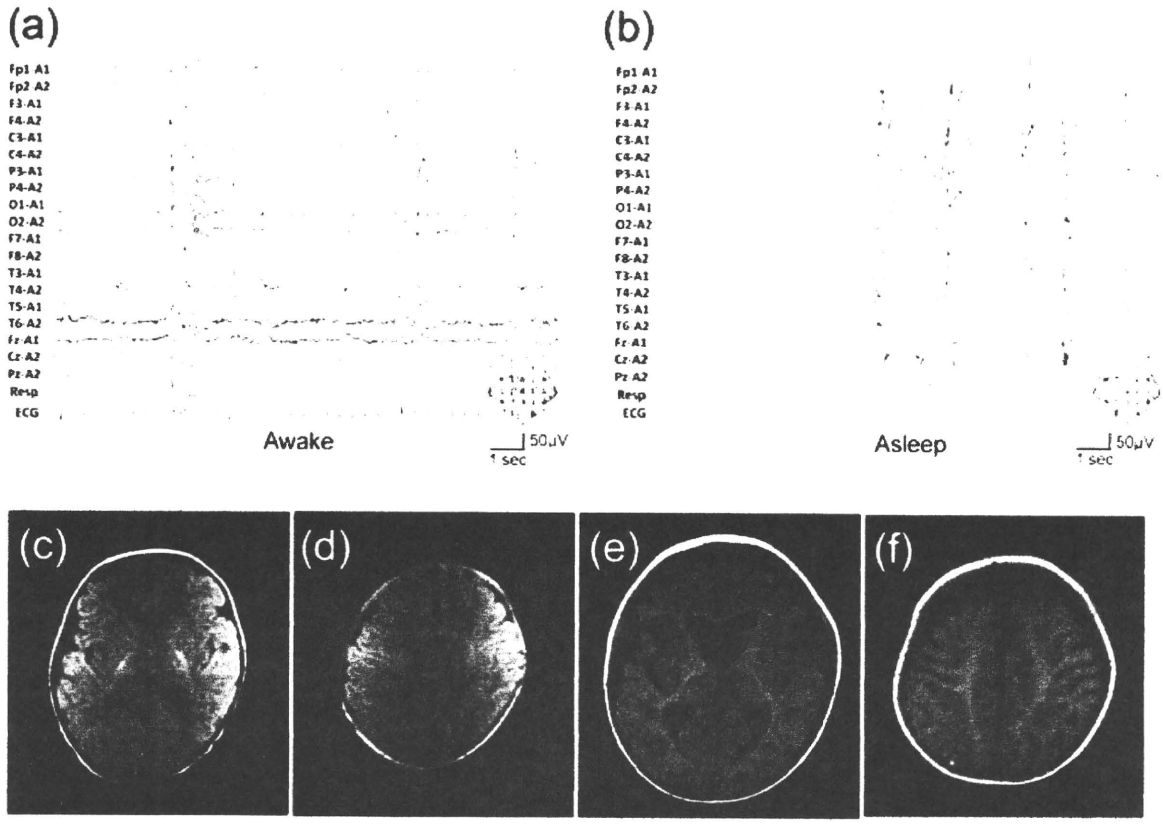

Fig. 1. Interictal electroencephalogram (EEG) (**a**, **b**) and brain magnetic resonance imaging (MRI) (**c**–**f**) of the patient. EEG during both waking (**a**) and sleep (**b**) at 1 month of age showed suppression-burst pattern consisting of low-voltage, almost flat phase and high-voltage paroxysmal activity phase. Brain MRI showed normal findings at 1 month of age (**c**, **d**), and slightly dilated lateral ventricles at 11 months of age (**e**, **f**) because of adrenocorticotropic hormone injection.

could affect donor splicing of exon 10, reverse transcriptase (RT)–PCR designed to amplify exons 9–12 was performed using total RNA extracted from LCL derived from the patient (Fig. 2a). A single band (286 bp), corresponding to the wild-type *STXBP1* allele, was amplified using a cDNA template from a control LCL (Fig. 2b). By contrast, a longer band was detected from the patient's cDNA (Fig. 2b). The longer mutant transcript had a 138-bp insertion of intron 10 sequences (Fig. 2c), producing a premature stop codon at amino acid position 302; therefore, the mutant mRNAs are probably to be degraded by NMD (11, 12). The intensity ratio of the mutant compared to the normal band was increased up to 36.3% after treatment with 30 μM cycloheximide, which inhibits NMD, compared to 13.8% in the untreated condition (Fig. 2d). Thus the mutant transcript suffered from degradation by NMD, which would result in haploinsufficiency of *STXBP1*.

To examine whether the c.902+5G>A mutation occurred *de novo*, the parental DNA extracted from whole blood were analyzed by HRM. Compared with the mother's sample, the patient's sample showed clearly shifted melting curve, indicating that the heterozygous c.902+5G>A mutation could be surely detected (Fig. 3a). Interestingly, the father's sample showed a slightly shifted melting curve, suggesting that the father may harbor the mutation in mosaic state, which was suggested by sequencing (Fig. 3a,b). Similar melting curves and electropherograms were obtained in DNA extracted from saliva, buccal cells, and nails (Fig. 3a,b). We further investigated the mosaicism by counting wild-type G and mutant A alleles after TA cloning of the PCR product. DNA extracted from blood, saliva, buccal cells, and nails suggested that 5.3%, 8.7%, 11.9%, and 16.9% of alleles (i.e. 10.6%, 17.4%, 23.8%, and 33.8% of cells) harbored the mutation, respectively (Fig. 3c).

Discussion

To date, 13 point mutations and one deletion of *STXBP1* have been reported in individuals with OS (9, 10). Thirteen out of fourteen deletion/mutations were confirmed as *de novo* events (paternal DNA was unavailable for one

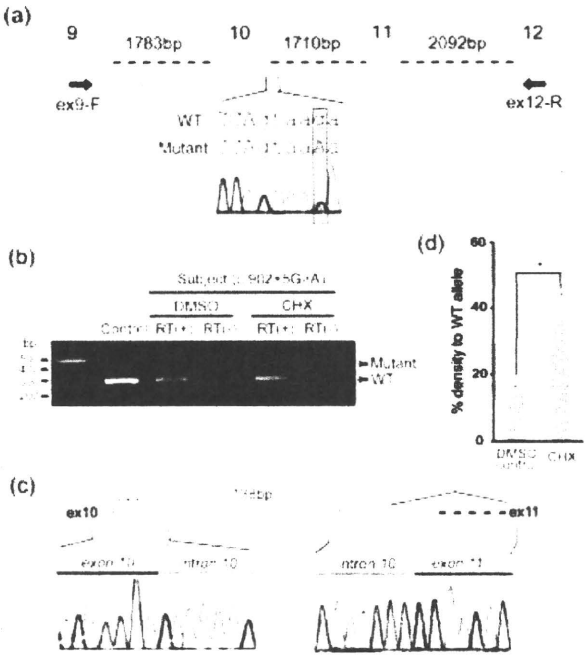

Fig. 2. **The c.902+5G>A mutation causing abnormal splicing associated with nonsense-mediated mRNA decay (NMD). (a)** Schematic representation of the genomic structure from exons 9 to 12 of *STXBP1*. Exons, introns and primers are shown by gray boxes, dashed lines and arrows, respectively. **The mutation in intron 10 was colored in red. Sequences of exon and intron are presented in upper and lower cases, respectively. (b)** Reverse transcriptase (RT) – PCR analysis of the patient with c.902+5G>A and a normal control. Two PCR products **were detected from the patient's cDNA: lower was the wild-type (WT) transcript and upper was the mutant. Only a single** WT amplicon was detected in a control. The mutant amplicon was significantly increased by 30-μM cycloheximide (CHX) **treatment compared to DMSO treatment as a vehicle control. RT (+): with reverse transcriptase, RT (−): without reverse** transcriptase as a negative control. **(c)** Sequence of mutant amplicons clearly showed a 138-bp insertion of intron 10 sequences and a premature stop codon (asterisk) in the mutant **transcript. (d) Quantitative analysis of the NMD inhibition by CHX based on the data shown in (b).** $*p = 0.00186$ by unpaired Student's *t*-test (two-tailed). Averages of duplicated experiments using two distinctive RNA samples, respectively, **are shown with error bars (standard deviation).**

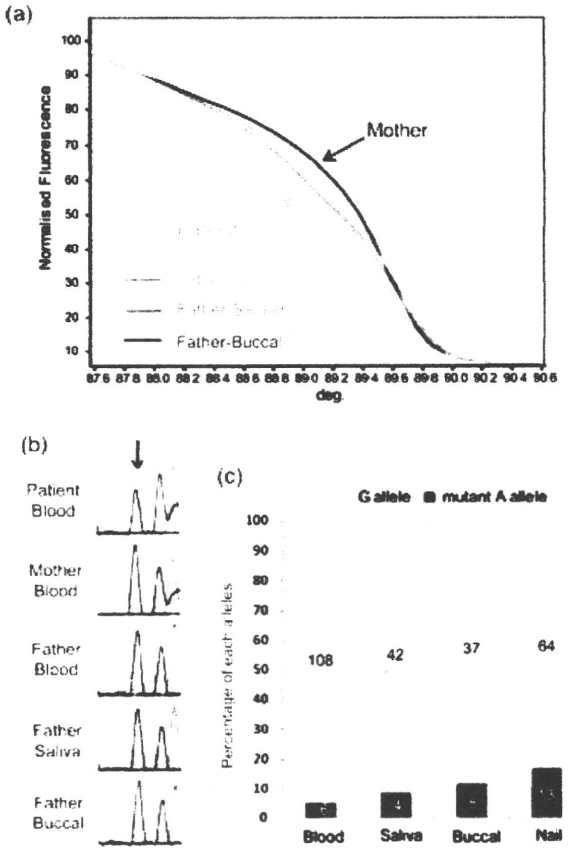

Fig. 3. Paternal somatic mosaicism of the c.902+5G>A mutation. **(a) Melting curves of PCR products. Compared with the mother's sample (black), the patient's sample (gray) showed** largely shifted melting curve. The father's sample from blood (red), saliva (green), and buccal cells (blue) showed slightly, but distinctly shifted melting curves. **(b)** Electropherograms of **the c.902+5G>A mutation (arrow) showed mosaicism of the mutation in the father. (c) Allele frequencies counted by TA** cloning of PCR products and sequencing. DNA extracted from blood, saliva, buccal cells, and nails of the father showed that **5.3%, 8.7%, 11.9%, and 16.9% of alleles harbored the mutant A allele. The numbers of colonies corresponding to each allele** are indicated within bars.

remaining mutation). Many OSs are sporadic, probably because of their poor outcome with severe psychomotor retardation; however, some X-linked familial cases have been reported with *ARX* mutations (6, 8). Here we have showed a paternal somatic mosaicism of an *STXBP1* mutation. Although DNA from the semen of the father could not be analyzed in this study, the identical c.902+5G>A mutation found in both the father and the affected daughter indicated that the father should possess the mutation in germ cells as a mosaic state, suggesting recurrence risks. Thus, somatic and germline mosaicism of *STXBP1* mutations should be carefully taken into account especially for genetic counseling of familial OS cases.

We have successfully identified the paternal somatic mosaicism of an *STXBP1* mutation by HRM. DNA from blood indicated that the mosaic ratio is as low as about 5%; therefore, HRM could be very sensitive in detecting low-ratio mosaicism. HRM is a rapid and simple approach to detect heteroduplexes (13). It only requires the addition of a saturating dye before PCR. By HRM analysis of the PCR products, the sensitivity of successful detection of heterozygotes is nearly 100% (13). It should be noted that the sensitivity of HRM to detect somatic changes or heteroplasmy is much

better than that of DNA sequencing (14, 15): HRM could detect the level of somatic mosaicism down to 5–10% (15). However, the ability to detect low percentage heterodeuplex of PCR products may vary among mutations. Although the heterozygous c.902+5G>A mutation showed largely shifted melting curve, we experienced some heterozygous mutations only showing slightly shifted melting curve, in which we may not be able to detect the mosaicism. Therefore, optimization of HRM analysis for each mutation would be recommended especially to examine parental samples.

In conclusion, we firstly described the paternal somatic mosaicism of an *STXBP1* mutation. The percentage of mosaicism was quite low (5–17%), and no minor problems like dexterity, intelligence (cognition), behavior or psychological state were recognized in the father. The information described here was quite useful for future genetic counseling of this family.

Acknowledgements

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RESEARCH ARTICLE Open Access

A robust method for estimating gene expression states using Affymetrix microarray probe level data

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Abstract

Background: Microarray technology is a high-throughput method for measuring the expression levels of thousand of genes simultaneously. The observed intensities combine a non-specific binding, which is a major disadvantage with microarray data. The Affymetrix GeneChip assigned a mismatch (MM) probe with the intention of measuring non-specific binding, but various opinions exist regarding usefulness of MM measures. It should be noted that not all observed intensities are associated with expressed genes and many of those are associated with unexpressed genes, of which measured values express mere noise due to non-specific binding, cross-hybridization, or stray signals. The implicit assumption that all genes are expressed leads to poor performance of microarray data analyses. We assume two functional states of a gene - expressed or unexpressed - and propose a robust method to estimate gene expression states using an order relationship between PM and MM measures.

Results: An indicator 'probability of a gene being expressed' was obtained using the number of probe pairs within a probe set where the PM measure exceeds the MM measure. We examined the validity of the proposed indicator using Human Genome U95 data sets provided by Affymetrix. The usefulness of 'probability of a gene being expressed' is illustrated through an exploration of candidate genes involved in neuroblastoma prognosis. We identified the candidate genes for which expression states differed (un-expressed or expressed) when compared between two outcomes. The validity of this result was subsequently confirmed by quantitative RT-PCR.

Conclusion: The proposed qualitative evaluation, 'probability of a gene being expressed', is a useful indicator for improving microarray data analysis. It is useful to reduce the number of false discoveries. Expression states - expressed or unexpressed - correspond to the most fundamental gene function 'On' and 'Off', which can lead to biologically meaningful results.

Background

Microarray technology is a high-throughput method for measuring the expression levels of thousand of genes simultaneously. Recent completion of the MicroArray Quality Control (MAQC) project ensures intra-platform consistency across test sites as well as a high level of inter-platform concordance [1]. As a result, microarrays are increasingly being used in the medical and biological fields as a powerful tool for disease diagnosis, identifying biomarkers, and studying gene function. However, observed intensities combine non-specific bindings including cross-hybridization or stray signals, which is a major disadvantage of microarray data.

The Affymetrix GeneChip microarray, in which Oligonucleotides of 25 bp are used to probe genes, is designed to include measures that allow the evaluation of non-specific hybridization. Each gene will be represented by 11~20 pairs of olligonucleotides referred to as a probe set (for example, the Human Genome U95 array uses 16 probe pairs and the Human Genome U133 Plus 2.0 array uses 11 probe pairs). Each of the probe pairs in a probe set consists of a perfect match (PM) and a mismatch (MM) probe. The PM probes are designed to bind perfectly to the gene of interest and the MM probes are created by changing the middle (13th) base to disrupt the bulk of specific hybridization [2]. However, opinions vary regarding the usefulness of MM measures.

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Background correction algorithms for the Affymetrix GeneChip microarray may be classified into two groups: those that use MM measures (e.g., dChip difference mode [3] as well as MAS5 [4] and its later, improved version *PLIER* [5]) and those that do not (dChip PM mode [6], RMA (Robust Multi-array Analysis) [7] and its modified version, *PM-only GC-RMA [8]*). *RMA and MAS5 are* representative algorithms used for background correction. With the RMA method, only PM is used to obtain a corrected intensity. MAS5 was originally provided as a default measure by Affymetrix, in which PMs are corrected by subtracting MMs, but many researchers pointed out that direct subtraction of MM from PM is unlikely to be useful [9]. The preprocessing step affects the stochastic properties of the final statistical summaries [10]. Biologists who want to analyze microarray data might be bewildered with the availability of so many preprocessing procedures with varying results [11].

Biologically, it is likely that not all observed intensities are associated with expressed genes -- that is, many of those are associated with unexpressed genes, of which measured values simply express noise due to non-specific binding, cross-hybridization, or stray signal [12]. It has been reported that only 30-40% of the genes [13] -- around 10,000-15,000 genes in total [14]-- are expressed in human cell lines *in vitro*. Identifying probe sets associated with un-expressed genes would allow the subsequent statistical analysis to be carried out with greater efficiency. For example, in an analysis aimed at finding differentially expressed genes, filtering out these probe sets prior to analysis contributed to a decreased number of false discoveries [12,15].

In previous work, we proposed a mathematical model based on the assumption that a gene has two separate functional states - 'On' means a gene is really expressed and 'Off' means a gene is un-expressed - for identifying differentially expressed genes between two cell types [16]. Furthermore, we proposed to identify 'Off' genes using an order relationship between PM and MM measures using Affymetrix GeneChip probe level data [17]. We applied the 'On/Off' model to real medical or biological data and obtained meaningful results [18-20]. In this study, we propose to quantify a gene as being expressed using a Weibull-Normal mixture distribution with two components corresponding to the separate states 'On' and 'Off'. The probability of a gene being 'On' is obtained from the posterior probability using this Weibull-Normal mixture distribution. We examine the advantage of our method over the detection call of MAS5 using the data sets of Human Genome U95 provided by Affymetrix. We implement our proposed methods of microarray analysis to explore candidate genes involved in neuroblastoma prognosis.

The symbol X denotes the number of pairs in a probe set satisfying $PM > MM$: $X = \#\{j | PM_j > MM_j, j = 1, \cup, J\}$, where J is the number of probe pairs in a probe set.

Results

Estimation of Weibull-Normal density function

Figure 1 illustrates the relationship between gene expression level and the value of X, where the RMA summarized value was used as the measure of gene expression level (signal intensity). It shows that a gene with high expression level has larger X -- that is, the gene is in the 'On' state. Similarly, a gene with small X ('Off' gene) has low expression intensity. However, not all genes with large X ('On' genes) evidence high expression levels. Figures 2A-C show PM and MM measurements for probe sets in which summarized expression levels are high, moderate, or low, respectively. Each probe set was sampled randomly from the high, moderate, or low expression group. If gene expression level is high enough, the PM value is adequately larger than the MM value in every probe pair, and it is possible in principle to separate a signal of specific binding from one of non-specific binding (Figure 2A). In the case that the MM value is close to the PM value in every probe pair, it is presumably difficult to separate a signal of specific binding from one of non-specific binding (Figure 2B). However, the value of X informs as to whether a gene is truly expressed or not. Figure 2B-1 shows an 'On' gene ($X = 8$) and Figure 2B-2 shows an 'Off' gene ($X = 3$). When gene expression intensity is low, it is difficult to distinguish non-specific signal from total signal intensity (Figure 2C). Figure 2C-1 shows an 'On' gene ($X = 10$) with low intensity. Figure 2C-2 shows an 'Off' gene ($X = 6$) with low intensity. In this case, both PM and MM values represent measures of non-specific binding. Briefly, the value of X provides qualitative information as to whether a gene is being expressed or not and it is more informative, especially when the gene expression level is not high. We propose to quantify a gene as being expressed using a random variable Z derived from X and assume that Z follows a Weibull-Normal mixture distribution with two components corresponding to the separate states 'On' and 'Off'. The probability of a gene being 'On' is obtained from the posterior probability using this Weibull-Normal mixture distribution.

The results of applying the Weibull-Normal mixture model to the Human Genome U95 data sets are shown in Figure 3. The estimated parameter vector was $(\mu, \alpha, \xi, \widehat{\sigma^2})$ = (1.00, 1.00, 0.35, 0.15), where μ and α denote location and power parameters of the Weibull distribution, ξ denotes mixture rate of 'Off' genes, and σ^2

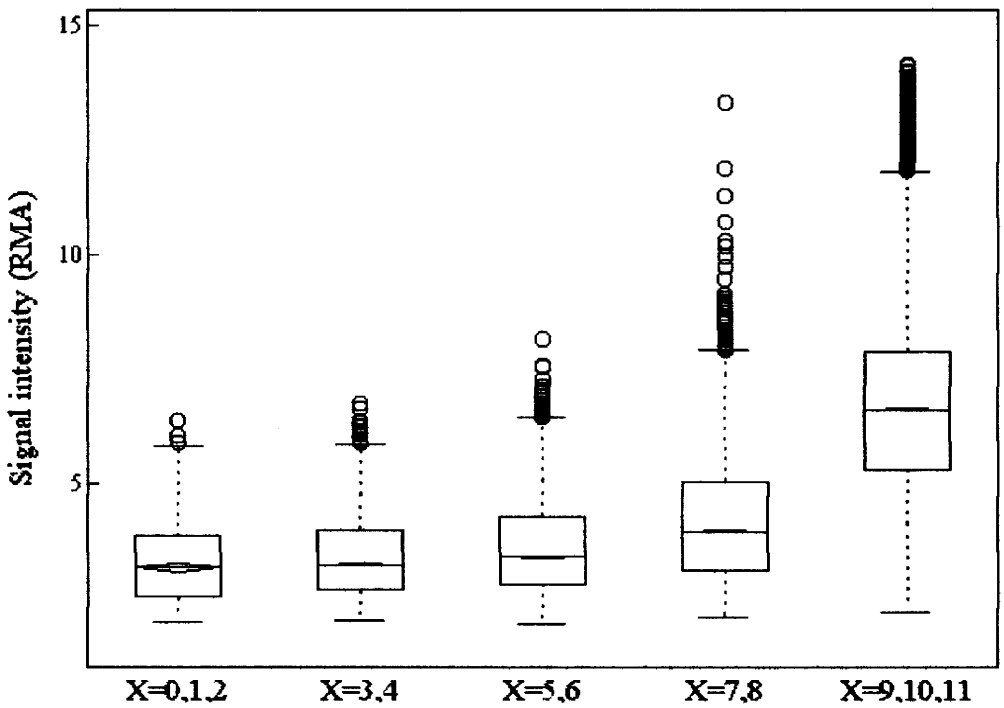

Figure 1 Parallel boxplots of gene expression levels by RMA graded according to the value of *X* (number of pairs where PM>MM). A gene with high expression level has a large value of *X*, but large *X* does not necessary imply a high expression level; expression level of a gene with large *X* can vary from low to high.

denotes the variance of the Normal distribution. Figure 3A shows a comparison of the fitted Weibull-Normal distribution with two components ('On' and 'Off') to the empirical distribution. Figure 3B shows the corresponding density function and its components. We defined the gene state as 'On' if $X \geq 11$ and 'Off' if $X \leq 10$. The vertical dotted lines in Figures 3A and 3B correspond to $X = 11$.

Comparison between MAS5 calls and 'On/Off' calls usingspike-in genes

The MAS5 method also provides a qualitative evaluation by calling gene expression present (P), marginal (M), or absent (A) for each probe set in determining whether the measured transcript is detected or not detected. However, there is an important difference between a gene being 'Off' and a call of 'absent'. In the cases of Figure 2B-1 and 2C-1, for example, the probe sets were called 'absent' whereas their states were determined to be 'On'. To make the detection call, the MAS5 method uses a nonparametric statistical test (Wilcoxon signed rank test) under the null hypothesis that PMs and MMs have the same distribution [4]. The MAS5 method attempts to identify truly expressed genes with certainty. Exclusion probes that are called 'absent' can result in many false negatives and loss of a large amount of information, especially with genes that switch between 'On' and 'Off' with different phenotypes. On the other hand, our method seeks to correctly identify 'Off' genes using an order relationship between PM and MM measures.

We compared 'On/Off' calls with the MAS5 calls using spike-in genes of the Human Genome U95 data sets. The spike-in genes with 0 pM concentration were used as negative controls (N = 59). The spike-in genes with more than 0.25 pM concentration were used as positive controls (N = 767). A cutoff point dividing gene states into 'On' and 'Off' was determined as the minimum value of X that contains as small an 'Off' component as possible using the fitted Weibull-Normal distribution (see 'Methods'). The value $X = 11$ was obtained as the cutoff point and is shown by the vertical dotted lines in Figures 3A and 3B. Table 1 shows the distribution of number of P/M/A calls by MAS5 and number of On/Off genes for each concentration of spike-in genes. As is shown in Table 2, MAS5 calls generated many false negatives (19.0%) compared to 'On/Off' calls (8.7%). 'On/Off' calls generated

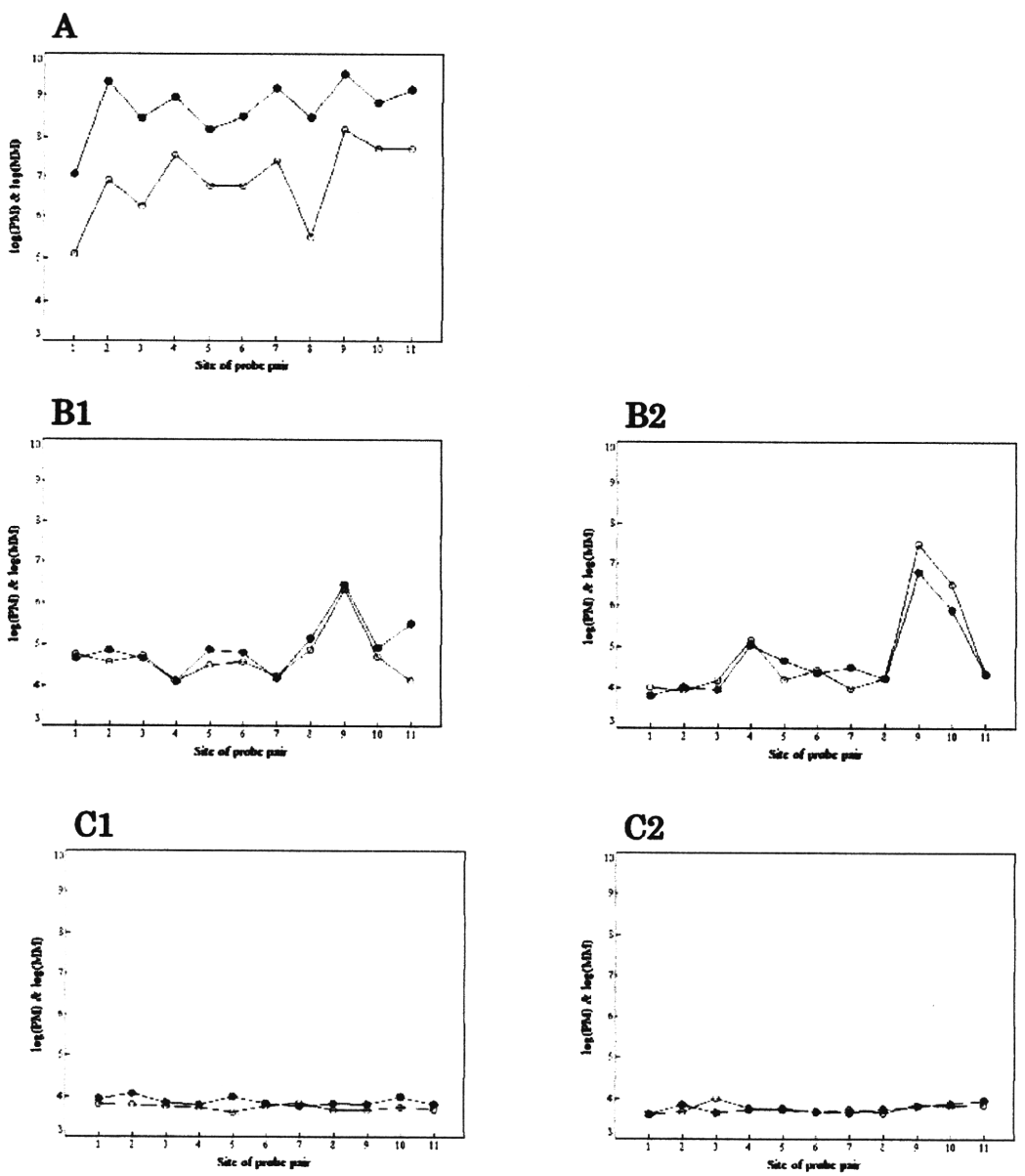

Figure 2 Comparison of PM and MM measurements within a probe set. Closed and open circles represent PM and MM measures, respectively. **(A)** High expression level. The PM value is suitably larger than MM value in every probe pair. Separating the signal of specific binding from that of non-specific binding might be possible in such a case. **(B)** Moderate expression level. The MM value is near the PM value in every probe pair; separating the signal of specific bindings from that of non-specific binding is difficult. However, the value of X is informative as to whether a gene is truly being expressed or not. (B1) shows 'On' ($X = 8$)) and (B2) shows 'Off' ($X = 3$). **(C)** Low expression level. It is difficult to determine whether a gene is expressed or not. (C1) shows 'On' ($X = 10$) and (C2) shows 'Off' ($X = 6$).