A comprehensive analysis of the *CDKN2A* gene in childhood acute lymphoblastic leukaemia reveals genomic deletion, copy number neutral loss of heterozygosity and association with specific cytogenetic subgroups.

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Running title: *CDKN2A* deletion in childhood ALL

ABSTRACT

Inactivation of the tumour suppressor gene, *CDKN2A*, can occur by deletion, methylation or mutation. We assessed the principal mode of inactivation in childhood ALL and frequency in biologically relevant subgroups. *CDKN2A* mutation or methylation was rare whereas genomic deletion occurred in 21% BCP-ALL and 50% T-ALL patients. SNP arrays revealed copy number neutral (CNN) LOH in 8% patients. Array CGH (aCGH) demonstrated that the mean size of deletions was 14.8Mb and biallelic deletions comprised a large and small deletion (mean sizes 23.3Mb & 1.4Mb). Among 86 patients tested by FISH and SNP/aCGH, only two small deletions (0.025Mb & 0.05Mb) were below the resolution of detection by FISH. Patients with high hyperdiploidy, *ETV6-RUNX1* or 11q23/*MLL* rearrangements had low rates of deletion (11%, 15%, 13%) whereas patients with t(9;22), t(1;19), *TLX3* or *TLX1* rearrangements had higher frequencies (61%, 42%, 78% and 89%). In conclusion, *CDKN2A* deletion is a significant secondary abnormality in childhood ALL strongly correlated with phenotype and genotype. The variation in the incidence of *CDKN2A* deletions by cytogenetic subgroup may explain its inconsistent association with outcome. The discovery of CNN LOH without apparent *CDKN2A* inactivation suggests the presence of other relevant genes in this region.

INTRODUCTION

Genetic alterations including chromosomal translocation, promoter hypermethylation, somatic mutation and gene deletion are believed to play a key role in oncogenesis. Alterations of the 9p21 locus have been implicated in many types of cancer, indicating a role for the tumour suppressor genes *CDKN2A* (*MTS1*) and CDKN2B (*MTS2*), which encode for $p16^{INK4a}$/$p14^{ARF}$ and $p15^{INK4b}$, respectively.[1] Loss of cell proliferation control and regulation of cell cycle are known to be critical to cancer development.[2] Both $p16^{INK4a}$ and $p15^{INK4b}$ specifically inhibit cyclin/CDK-4/6 complexes that block cell division during the G1/S phase of the cell cycle.[3]

It has been reported that *CDKN2A* and *CDKN2B* are frequently inactivated in various haematological malignancies.[1,4] Loss of heterozygosity (LOH) of chromosome arm 9p, including the *CDKN2A* locus, is one of the most frequent genetic events in childhood acute lymphoblastic leukaemia (ALL) suggesting inactivation of the second allele or, possibly, haplo-insufficiency.[5-8] Haplo-insufficiency of a tumour suppressor gene, e.g. *CDKN2A*, has been shown to be adequate to promote tumour progression.[9-11] Homozygous deletion of *CDKN2A* has been suggested as the dominant mechanism of its inactivation in leukaemogenesis.[12] However, the reported frequencies

of both hetero- and homozygous deletions in childhood ALL vary, 9-27% and 6-33% in B-cell precursor (BCP) ALL and 7-18% and 30-83% in T-ALL, respectively.[13] Similarly, the frequency of hypermethylation of the *CDKN2A* promoter has been reported to vary from 0-40% in childhood ALL.[14-19] Although mutations in exons 1 and 2 of *CDKN2A* have been described in childhood ALL, their incidence appears to be low, ranging from 0-7%.[12,20-24]

As reported data on *CDKN2A* alterations in childhood ALL are discrepant, it remains important to reveal the role of this gene in cancer development. In this study we have used mutation and methylation analyses as well as genomic technologies to elucidate the principal mode of *CDKN2A* inactivation in childhood ALL. Moreover, the use of array based comparative genomic hybridisation (aCGH) and single nucleotide polymorphism (SNP) microarray mapping has allowed the architecture of *CDKN2A* deletions in this disease to be characterised. Finally, extensive screening using fluorescence *in situ* hybridisation (FISH) has enabled the frequency of deletions to be accurately determined in biologically relevant subgroups.

MATERIALS AND METHODS

Patient samples

Diagnostic or relapse samples were obtained from patients entered to a NCRI Childhood Cancer and Leukaemia Group (CCLG) treatment trial (UKALLXI, ALL97, or ALL2003) or treated locally within the Northern Region of the UK after informed consent was obtained in accordance with the Declaration of Helsinki. All patients were diagnosed between 1986 and 2007. Cytogenetic and FISH data from diagnostic and relapse samples were collected either by the LRF UKCCG Karyotype Database[25] or the NHS Northern Genetics Service. Institutional Review Board approval was provided by each participating institution. Peripheral blood lymphocytes were obtained from a healthy individual for use as a normal control. Genomic DNA was extracted from mononuclear cells after density gradient centrifugation using a commercial kit (QiAmp DNA Blood Kit, Qiagen, USA), according to the manufacturer's instructions.

Mutation analysis

PCR amplification for each exon was performed in 50 µl volume assays containing 1 X PCR buffer, 200 µM of dNTP mix, 1.5-2.5 mM MgCl2, 0.2-0.4 µM of each primer, 1.25U of Amplitaq Gold polymerase and 100 ng of genomic DNA. In addition, a final concentration of 2.5% dimethyl sulfoxide (DMSO) was used in the exon 2 reaction mixture. Primer pairs for exon 1, 2 and 3 were

previously described.[20,26] PCR conditions for exon 1 and 2 consisted of initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds. The final extension at 72°C was performed for 5 minutes. PCR amplification of exon 3 was carried out using Touchdown protocol consisting of initial denaturation at 95°C for 10 minutes, followed by 14 cycles (20 sec denaturation at 94°C, 1 min annealing at 65-58°C with a decrease of 0.5°C every cycle and 1 min extension at 72°C) and a further 20 cycles with annealing at 58°C for 1 min. All PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized under UV transillumination

Mutation screening of all exons was performed using denaturing high performance liquid chromatography (dHPLC) (WAVE® Nucleic Acid Fragment Analysis System, Transgenomic, UK). All PCR products showing homoduplex profiles were spiked with approximately equimolar proportions of a product known to contain a wild-type *CDKN2A* fragment, and re-screened in order to differentiate between wildtype homozygote and homozygote variants. DNA from a melanoma cell line (MM384) kindly provided by Dr. Nicholas Hayward (Queensland Institute of Medical Research, Australia) and a myeloid leukaemia cell line (HL60), known to contain *CDKN2A* mutations were used as positive controls for exon 1a and 2, respectively. Heteroduplex profiles for MM384 and HL60 cell lines were detected after spiking consistent with their homozygous mutant status. All samples showing an altered dHPLC profile were directly sequenced or cloned and then sequenced using standard methods (Pinnacle Lab., Newcastle University, UK).

Methylation analysis

The methylation status of the *CDKN2A* promoter regions was assessed by methylation specific-PCR (MSP) analysis. DNA modification (bisulphite treatment) was conducted by using CpGenome DNA Modification Kit (Chemicon International, CA). A total of 250 ng DNA was treated according to the manufacturer's protocol. DNA from Raji and HL60 cell lines were used as controls for methylated and unmethylated *CDKN2A*, respectively, as previously described.[3] All bisulphite-treated DNA samples were amplified by using the primer sets specific for the unmethylated and methylated *CDKN2A* sequences (referred to as p16U and p16M respectively), as previously reported.[27] The final concentration of the reagents in the PCR mixture were 1 X PCR buffer, 200 µM dNTP mix, 1.5 mM MgCl2, 0.2 µM of each primer set for p16U and p16M, 1.25U of Amplitaq Gold polymerase and bisulphite-modified DNA (50 ng) in a final volume of 25 µl. PCR conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 14

cycles (20 sec denaturation at 94°C, 1 min annealing at 64-57°C with a decrease of 0.5°C every cycle and 1 min extension at 72°C) and a further 32 cycles with annealing at 57°C for 1 min. Each PCR product was directly loaded onto 1.8% agarose gel, stained with ethidium bromide and visualised under UV transillumination. MSP analysis was repeated for each patient sample to confirm the methylation status of *CDKN2A*.

Single nucleotide polymorphism mapping array (SNPA)

DNA samples at relapse and presentation were analysed using a GeneChip Human Mapping 10K and/or 50K array (Affymetrix UK Ltd, High Wycombe, UK) to characterize LOH and allele copy number. Each SNP on the array is represented by 40 different 25bp oligonucleotides, each with slight variations which allow accurate genotyping. Sample analysis was performed by MRC Geneservice (Hinxton, Cambridge, UK). Data from microarray analysis were analysed using the Copy Number Analyser for Affymetrix® GeneChip (CNAG) software package (version 1.1), to identify regions of chromosomal deletion, amplification or LOH.[28]

Array based comparative genomic hybridisation (aCGH) analysis

aCGH was performed with array platforms constructed from either large-insert DNA clones or oligonucleotides, offering genome coverage at 1Mb and 6Kb intervals, respectively. For the large insert arrays, DNA samples were processed in a dye-swap combination and sex-matched with DNA extracted from phenotypically normal individuals (Promega, Southampton, UK) before hybridization to two commercially available systems (Spectral Genomics and IntegraGen, Genosystems, Paris, France), as previously reported.[29] For the oligonucleotide array, sample DNA was sex-matched to the same control DNA and hybridized to the 244K whole genome array (Agilent Technologies, Santa Clara, California, USA) according to manufacturer's instructions.[30] Scanning, grid placement, spot quality, data normalization, fluorescence quantification, post-processing of the array image and data analysis were performed as previously described.[31,32] Regions of copy number alteration (CNA), identified by the large-insert array, were defined as a minimum of three adjacent clones simultaneously deviating beyond the threshold values in both dye-swap experiments and were positioned onto the human genome sequence using BlueFuse software (BlueGnome, Cambridge, UK). For the oligonucleotide array analysis, CGH Analytics software (Agilent Technologies, Santa Clara, California, USA) was used to identify regions of CNA based on the z-score and mathematical aberration detection analysis (ADM1, Agilent Technologies).

Fluorescence in situ hybridization (FISH)

FISH analysis was performed on frozen cytospins and/or fixed cells from diagnostic bone marrow. Three commercial probes were used to detect *CDKN2A* deletions in leukaemic cells: LSI® p16 (9p21) SpectrumOrange™/CEP® 9 SpectrumGreen™ Probe (Vysis/Abbott Diagnostics, Maidenhead, UK); P16 Deletion Probe (Cytocell Technologies Ltd., Cambridge, UK); p16 (*CDKN2A*) specific probe (Qbiogene/MP Biomedicals, Cambridge, UK). Probe and slide preparation as well as hybridization and washing steps were performed according to the manufacturers' protocols. An additional home-grown dual-colour, *CDKN2A* deletion probe (consisting of BAC RP11-149I2/70L8 and the chromosome 9 centromere probe pMR9A; all clones from Sanger Institute, Hinxton, UK) (Wessex Regional Genetics Laboratory, WRGL) was also used. These were grown and labelled for FISH as previously described.[33] The approximate size of the *p16/CDKN2A* probes are: Vysis - 190kb; Cytocell – 101kb; Qbiogene – not available; WRGL – 101kb. A minimum of 100 interphase cells were scored in each test. A signal pattern of two red and two green (2R2G) was considered to represent a normal cell. Loss of one signal corresponding to the test and retention of both signals for the control probe (i.e. 1R2G or 2R1G depending on the probe) was considered to represent a heterozygous deletion. A homozygous deletion was defined as loss of both test signals with retention of both control signals (i.e. 0R2G or 2R0G depending on the probe). The cut-off level defined to rule out a false positive result was calculated to be 5%. Therefore, only abnormal cell populations comprising more than 5% of analysed cells were included. Rare cases with two different deleted populations (one heterozygous and one homozygous) were classified as having a homozygous deletion. Cases with loss of a whole chromosome 9 were not classified as having a deletion.

RESULTS

Mutation screening

Mutation screening of all three exons was successfully performed on 47 patients who were known to have retained (by FISH or SNPA) at least one *CDKN2A* allele. Samples tested were from diagnosis (n=21), relapse (n=25) or both time points (n=1) (Table 1). Chromatography profiles generated from dHPLC were inspected to detect heteroduplexes which indicated the presence of mutations/polymorphisms. All aberrant profiles were confirmed by DNA sequencing analysis. A known SNP was detected in 15 (32%) patients; detected at diagnosis (n=4), relapse (n=10) or both time points (n=1). Nine (19%) patients had the 500C>G SNP in the 3'UTR of exon 3: 7

(15%) patients were CG heterozygotes while 2 (4%) were GG homozygotes. Three patients who were CG heterozygotes at nucleotide 500 also carried the A148T polymorphism in exon 2. Six (13%) patients had the 540C>T polymorphism in the 3'UTR of exon 3: 4 (9%) patients were CT heterozygotes while 2 (4%) were TT homozygotes. Only one patient (L5C) harboured a somatic mutation. This was detected in a relapse sample and involved a base substitution of C>T in exon 2 at nucleotide 247, which resulted in the amino acid tyrosine replacing histidine at codon 83 (H83Y). Although this relapse sample showed an aberrant profile after dHPLC, initially no mutation was detected by sequencing analysis. DNA from this patient was cloned and after re-sequencing the mutation was confirmed in 2/20 clones; suggesting that the quantity of mutant DNA was too low to be detected by first direct sequencing. We concluded that only a minority of the blast cells harboured this mutation which was detected by the sensitive dHPLC technique but not by direct sequencing, in agreement with a previous study.[34] This patient had a normal karyotype and did not show loss of *CDKN2A* by FISH. However, SNP array analysis (see below) revealed copy number neutral LOH from 9p21.1 to the telomere.

Methylation screening

MSP analysis was performed on a total of 99 patients who were known to have retained (by FISH or SNPA) at least one *CDKN2A* allele (Table 1). Samples tested were from diagnosis (n=70), relapse (n=26) or both time points (n=3). Each assay was performed twice and included a positive control (Raji cell line in which *CDKN2A* is methylated) and a negative control (HL60 cell line in which *CDKN2A* is unmethylated). The presence of a 151 bp and a 150bp product in the relevant lane indicated that the sample was umethylated (U) and methylated (M) respectively. None of the patients showed complete methylation of the *CDKN2A* promoter but one patient (L450) had partial methylation as shown by the presence of both U and M products. Partial methylation suggests the presence of a mixed population of blasts: one with and one without methylation of the *CDKN2A* promoter. This patient had a normal karyotype, did not have a mutation and had an intact 9p by aCGH (see below).

Genomic analyses

High density SNPA analyses were performed on 98 patients at diagnosis (n=78), relapse (n=12) or both time points (n=8) (Table 1). No abnormality of the short arm of chromosome 9 was detected in 73 (74%) patients whereas 25 (26%) patients harboured either a deletion (n=17) or CNN LOH (n=8) of 9p. The absence of a 9p deletion was confirmed in 27 patients by FISH (n=8),

aCGH (n=11) or both techniques (n=8). SNPA revealed a deletion of 9p in 17 (17%) patients (Figure 1Ai-iii). Most of the deletions were terminal [n=11: del(9)(p12)x1, del(9)(p13.1)x1, del(9)(p13.2)x1, del(9)(p13.3)x4, del(9)(p21.2)x1 and del(9)(p21.3)x3] but interstitial deletions also occurred [n=6: del(9)(p21.2p21.3)x1, del(9)(p21.3p22.1)x2, del(9)(p21.3p22.3)x1, del(9)(p21.3p21.3)x2]. All the deletions were confirmed by FISH (n=10), aCGH (n=2) or both techniques (n=5). In addition to these deletions, SNPA revealed CNN LOH (also known as acquired isodisomy and uniparental disomy) in 8 (8%) patients at diagnosis (n=6) and relapse (n=2). The area of CNN LOH (Figure 1Aiv) ranged from ~28Mb to the whole chromosome: 9p21.1->9pter (n=2), 9p13->9pter (n=1), 9q21->9pter (n=1) and whole chromosome 9 (n=4). None of these patients showed a *CDKN2A* deletion by FISH (n=7 tested) or promoter hypermethylation (n=8). However, one patient did harbour a H83Y mutation in exon 2 of *CDKN2A* (see above). A high hyperdiploid karyotype was detected in 5 of these 8 patients, including 3 of the 4 patients with whole chromosome 9 CNN LOH but 2 normal copies of chromosome 9 was seen by cytogenetics. Cytogenetics failed or was normal in the remaining 3 patients.

A selected series of patients (n=105) were analysed using aCGH at diagnosis (n=102), relapse (n=2) or both time points (n=1) (Table 1, Figure 1B). Overall 34 (32%) patients harboured a deletion of *CDKN2A*, including 15 which showed biallelic loss (Figure 2). FISH analysis confirmed the presence of a *CDKN2A* deletion in 19/20 (95%) patients tested. One patient (patient 10026) showed a very small (0.045Mb) monoallelic deletion of *CDKN2A* which was below the resolution of the commercial or home-grown FISH probes. In another patient (patient 2741), aCGH detected a large (36.7Mb) monoallelic deletion accompanied by a very small (0.025Mb) biallelic deletion, the latter of which was below the resolution of either FISH probe. The size of the deletions varied considerably from 0.025Mb to 39.1Mb, with a mean of 14.5Mb and median of 7.3Mb. Among 15 patients with biallelic deletions, a pattern of one large and one small deletion was evident (Figure 2). The mean size of the larger deletion was 23.3Mb whereas the smaller deletion was more focal with a mean size of 1.4Mb: 7/15 biallelic deletions were less than 1Mb.

Correlation of CDKN2A deletions with demographic and cytogenetic features

In order to assess the true incidence of *CDKN2A* deletions in childhood ALL as a whole and within relevant biological subgroups, an extensive interphase FISH screening programme was carried out. Three independent and unbiased cohorts were identified as follows: 864 diagnostic samples from

patients with BCP-ALL; 266 diagnostic samples from patients with T-ALL; and a relapse cohort comprising 69 BCP-ALL and 8 T-ALL patients. A total of 1,207 samples were screened with one of four FISH probes: Vysis, Cytocell, Qbiogene and WRGL (see methods and Table 1). However, the majority of samples (96%) were tested with either the Vysis probe (n=515), WRGL probe (n=870) or both (n=216). Among 226 samples tested with more than one FISH probe, only 11 (<5%) cases showed discrepant results. In 4 cases the WRGL probe detected a biallelic deletion where the Vysis probe had detected only a monoallelic deletion. In the other 7 cases, no deletion was detected by the Vysis probe whereas the WRGL or Qbiogene probe detected a monoallelic (n=5) or biallelic (n=2) deletion. These discrepancies are due to the fact that the Vysis probe is ~90kb larger than the WRGL or Qbiogene probes. Conventional cytogenetics was attempted on virtually all these samples (1197/1207, 99%) and was of sufficient quality to assess 9p status in 884 (73%). A cytogenetically visible 9p deletion/abnormality was detected in 133/277 (48%) samples where FISH detected a deletion. In a small number of samples (n=19) a cytogenetically visible 9p abnormality did not correlate with a deletion of *CDKN2A*.

The incidence of *CDKN2A* deletions and the proportion of biallelic deletions present at diagnosis were higher in T-ALL patients compared to BCP-ALL patients: 50% v 21% ($p<0.001$) & 62% v 43% ($p=0.001$), respectively (Table 2). Among BCP-ALL patients, *CDKN2A* deletions were more prevalent among older children (10+ years, $p<0.001$) and those with a white cell count (WCC) of greater than $50x10^9$/L ($p<0.001$). However, this was not the situation among T-ALL patients (Table 2). Moreover, within the BCP-ALL and T-ALL cohorts the incidence of *CDKN2A* deletions was associated with specific cytogenetic subgroups. The incidence of deletions was significantly lower among BCP-ALL patients with the *ETV6-RUNX1* fusion or high hyperdiploidy, whereas it was significantly higher among patients with t(9;22)(q34;q11) or t(1;19)(q23;p13) (Table 2). Among T-ALL patients, those with involvement of *TLX1* or *TLX3* had significantly higher rates of deletion when compared with T-ALL patients without these abnormalities (Table 2). Considering BCP-ALL and T-ALL patients together, *CDKN2A* deletions were rare among those with *MLL* translocations [4/33 (12%) v 285/1012 (28%), $p<0.05$]. However, the difference was not significant when BCP-ALL and T-ALL patients were considered separately (Table 2). Among 10 patients with t(4;11)(q21;q23) only one had a homozygous deletion of *CDKN2A* and none of the 9 patients with t(11;19)(q23;p13) harboured a deletion. Despite the incidence of *CDKN2A* deletions varying markedly by immunophenotype, age, WCC and cytogenetic subgroup, the proportion of biallelic deletions was relatively stable: 40% among BCP-ALL and 60% among T-ALL patients. Two possible

exceptions were t(9;22) patients with BCP-ALL and those with normal karyotypes, where monoallelic and biallelic deletions dominated respectively (Table 2).

The incidence of *CDKN2A* deletions at relapse was slightly higher than that seen at diagnosis among BCP-ALL patients, although the difference was not statistically significantly: 21/69 (30%) v 180/864 (21%) p=0.06. The relapse cohort comprised only 8 T-ALL patients of which only 2 (25%) harboured a *CDKN2A* deletion, however both were biallelic. Among 44 patients where we were able to assess *CDKN2A* status at both diagnosis and relapse by FISH, SNPA or aCGH, 28 (64%) patients had normal *CDKN2A* at both time points. The remaining 16 (36%) patients showed retention (n=9, 20%) or gain (n=7, 16%) of a *CDKN2A* deletion or 9p CNN LOH between diagnosis and relapse. The 7 *CDKN2A* abnormalities acquired at relapse comprised: 2 monoallelic deletions, 4 biallelic deletions and 1 CNN LOH between 9p21.1 and the telomere. These 7 patients did not have any remarkable features with respect to age at diagnosis, WCC, immunophenotype, karyotype or time to relapse (data not shown). Interestingly, 6 (86%) of these patients were male and three relapses involved the testes as well as the bone marrow.

DISCUSSION

This is the first study to utilise five separate technologies to assess the principal mode of *CDKN2A* inactivation in childhood ALL. We found that *CDKN2A* mutations were rare in this disease which agrees with most [21-24] but not all previous studies.[12,20] We found only one mutation which was a C to T substitution in exon 2 that produced an amino acid change from histidine to tyrosine at codon 83 (H83Y) and has been shown to be defective in inducing cell cycle arrest.[35] This H83Y mutation has not previously been reported in leukaemia but has been detected in bladder and gastrointestinal stromal tumors.[36,37] Despite screening over 100 samples for hypermethylation of the *CDKN2A* promoter, we found partial methylation in only one diagnostic sample. Two previous studies of a similar size found *CDKN2A* methylation in 7% [16] and 32% [18]. Several much smaller studies (<30 patients) have reported equally variable levels of methylation. However, it has been suggested recently that *CDKN2A* hypermethylation is not a disease specific event since it has been observed in the mononuclear cells from normal individuals.[38] In contrast, we have demonstrated that genomic deletion of *CDKN2A* is substantially more prevalent in childhood ALL (20% in BCP-ALL and 50% in T-ALL) than either mutation or methylation. As the cohorts we screened for mutation and methylation excluded cases with biallelic deletion of *CDKN2A* our figures are under-estimates of the true incidence. We assessed genomic deletion by SNPA, aCGH and FISH and

found a very high degree of concordance between the three techniques with only a handful of deletions detected by aCGH which were below the resolution of FISH. The apparent difference in detection rate between SNPA (17%) and aCGH (32%) is due to the fact that specific cytogenetic subgroups were selected for aCGH analysis. Therefore, we concluded that genomic deletion is the principal mode of *CDKN2A* inactivation in childhood ALL and that FISH is a reliable and accurate method for detecting these deletions.

In order to accurately assess the incidence of mono- and biallelic deletions in childhood ALL, we screened two large and unbiased cohorts of BCP-ALL and T-ALL patients by FISH. Over 200 patients were screened with more than one FISH probe and there was a high degree of concordance. As expected the smaller home grown WRGL probe detected a handful of smaller deletions that were below the resolution of the commercial probes. Despite these discrepancies and those detected when comparing aCGH and FISH, the numbers of patients involved were small and not sufficient to affect the subsequent analyses. In agreement with previous large studies,[13,39,40] we found that the incidence of *CDKN2A* deletions was significantly higher among T-ALL patients compared with BCP-ALL patients which included the proportion of biallelic deletions. Among BCP-ALL patients, we found a frequency of *CDKN2A* deletions of 21% which was the same as Kawamata et al[41] (21%) but lower than Bertin et al [13] (31%) and Mullighan et al [39] (34%). We found that 50% T-ALL harboured a *CDKN2A* deletion, a frequency substantially lower than that reported by Bertin et al [13] (78%), Mullighan et al [39] (72%) and Kawamata et al [41] (78%). These differences are probably due to variation in the underlying patient cohort with respect to age and WCC rather than due to the technique employed. Firstly, evidence from our study and Usvasalo et al [40] indicated that the vast majority of *CDKN2A* deletions are detectable by FISH. Secondly, data from this study and Mirebeau et al [42] showed that the frequency of *CDKN2A* deletions varied according to age and presenting WCC. Our study included over 1,200 patients (more than the combined total from the three main previous studies), thereby minimising the effect of any slight variations in age or WCC distribution.

The number of cases included in our study was sufficiently large to systematically address the relationship between *CDKN2A* deletions and specific cytogenetic subgroups. *CDKN2A* deletions were less frequent in patients with high hyperdiploidy and the *ETV6-RUNX1* fusion compared to those without these abnormalities. We found a similar incidence of deletions among high hyperdiploidy patients as Mirebeau et al [42], but these authors found that over a third of *ETV6-*

RUNX1 patients had a deletion. However their result was based on fewer than 50 patients, whereas over 200 *ETV6-RUNX1* positive patients were included in this study. In addition, we observed a higher incidence of *CDKN2A* deletions among patients with t(1;19) and t(9;22). Although patients with a *MLL* translocation were divided between the BCP-ALL and T-ALL cohorts, it was clear that the incidence of *CDKN2A* deletions was rare in this subgroup with only 4/33 (12%) showing evidence of a deletion. Interestingly the incidence also varied within the T-ALL cohort where patients with *TLX3* or *TLX1* rearrangements had a high incidence of deletion. In a separate study, we have recently observed a strong correlation between the presence of t(6;14)(p22;q32)/*IGH@-ID4* and a monoallelic *CDKN2A* deletion.[43]

As in previous studies, we observed both monoallelic and biallelic *CDKN2A* deletions and found that the latter were more prevalent in T-ALL.[13,40,42] Interestingly, within the BCP-ALL and T-ALL cohorts the proportion of biallelic deletions did not vary by age, WCC or cytogenetic subgroup, with the exception of t(9;22) and normal karyotypes. However these observations were based on relatively few cases. In our cohort, biallelic deletions comprised one large and one much smaller deletion. A recent aCGH/FISH study by Usvasalo[40], which focussed on older children and adolescents, observed a similar architecture.

The use of SNP arrays allowed the identification of CNN LOH, a phenomenon which has recently been associated with malignancy, including ALL.[41,44-46] Previous studies have shown that this genetic mechanism is associated with the presence of homozygous mutations: *JAK2* in myeloproliferative diseases, *PTCH* in basal cell carcinomas and *FLT3* in AML.[44,47,48] None of our *CDKN2A* CNN LOH cases were associated with deletion or methylation and only one had a *CDKN2A* mutation. However, the region of CNN LOH always encompassed numerous other genes and in half of the cases extended to the whole chromosome; indicating the likely involvement of other candidate genes such as PAX5 at 9p13.2.[39] Recently, Kawamata et al[41] identified a similar incidence of 9p / whole chromosome 9 CNN LOH (12%) among children with ALL. However, Kawamata et al found an association between 9p CNN LOH and *CDKN2A* deletion but not with whole chromosome 9 CNN LOH. Interesting both studies showed a strong association between 9p CNN LOH and high hyperdiploidy and this was particularly strong for cases with whole chromosome 9 CNN LOH. Therefore, it is not surprising that chromosome 9 is rarely gained in children with high hyperdiploidy.[49]

The literature is divided as to the prognostic relevance of *CDKN2A* deletions in childhood ALL with some studies indicating an adverse effect[50-54] while others show no effect.[42,55,56] These studies followed several cytogenetic based reports which suggested an adverse outcome for patients with a cytogenetically visible 9p abnormality.[57,58] The majority of patients in this study are being treated on an ongoing clinical trial, hence we are unable to present survival analysis. However, our results are highly relevant to this debate on prognosis. We have demonstrated unequivocally that the frequency of *CDKN2A* deletions is unevenly distributed across distinct cytogenetic subgroups and therefore any study assessing their prognostic impact must incorporate analysis stratified by cytogenetics. It is possible that the inconsistent results of previous studies with respect to the prognostic relevance of *CDKN2A* deletions are due to the variable frequency of deletions by cytogenetic subgroup. However, it is also possible that *CDKN2A* deletions do not represent a true independent prognostic factor. A large study incorporating cytogenetics and the accurate determination of *CDKN2A* deletion is required to address this important issue. We did not observe any difference in the incidence of *CDKN2A* deletion between diagnosis and relapse but given the small number of patients in our series a larger study will be required to definitely answer this question. However, we would not draw the same conclusion for T-ALL where the frequency of *CDKN2A* deletions is higher, less variable by genetic subtype and the subject of fewer analyses.

In conclusion we have demonstrated that the principal mode of *CDKN2A* inactivation in childhood ALL is by genomic deletion and that FISH is an efficient detection method. Moreover, we have established that the frequency of deletions varies considerably by genetic subtype, especially within BCP-ALL where this variation is likely to account for discrepant reports as to its prognostic relevance. The architecture of biallelic deletions and the observation of large areas of 9p with disease specific CNN LOH implicate other genes within this region which are likely to be important, both for maintaining and initiating the leukemia phenotype.

ACKNOWLEDGEMENTS

The authors would like to thank all member laboratories of the UKCCG for providing cytogenetic results, cell suspensions and/or cDNA (a full list of laboratories is available on request) and the Clinical Trial Service Unit (CTSU, University of Oxford, UK) for providing clinical data. We are grateful to Drs John Crolla and Fiona Ross and their teams at Wessex Regional Genetics Laboratory (WRGL, Salisbury, UK) for the growing and preparation of the home-grown *CDKN2A* and CTB-25B13 FISH probes. This study could not have been performed without the dedication

of the Children's Cancer and Leukaemia Group Leukaemia and Lymphoma Division (CCLG LLD) (previously known as the UK Childhood Leukaemia Working Party) and their members, who designed and coordinated the clinical trials through which these patients were identified and on which they were treated.

REFERENCES

1. Ruas M, Peters G. The p16(INK4a)/CDKN2A tumor suppressor and its relatives. Biochimica et Biophysica Acta. 1998;1378:F115-F177.
2. Sandal T. Molecular aspects of the mammalian cell cycle and cancer. Oncologist. 2002;7:73-81.
3. Drexler HG. Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. Leukemia. 1998;12:845-859.
4. Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene. 2002;21:3475-3495.
5. Baccichet A, Qualman SK, Sinnett D. Allelic loss in childhood acute lymphoblastic leukemia. Leukemia Research. 1997;21:817-823.
6. Takeuchi S, Koike M, Seriu T, et al. Homozygous deletions at 9p21 in childhood acute lymphoblastic leukemia detected by microsatellite analysis. Leukemia. 1997;11:1636-1640.
7. Takeuchi S, Tsukasaki K, Bartram CR, et al. Long-term study of the clinical significance of loss of heterozygosity in childhood acute lymphoblastic leukemia. Leukemia. 2003;17:149-154.
8. Irving JA, Bloodworth L, Bown NP, Case MC, Hogarth LA, Hall AG. Loss of heterozygosity in childhood acute lymphoblastic leukemia detected by genome-wide microarray single nucleotide polymorphism analysis. Cancer Res. 2005;65:3053-3058.
9. Komuro H, Valentine MB, Rubnitz JE, et al. p27KIP1 deletions in childhood acute lymphoblastic leukemia. Neoplasia. 1999;1:253-261.
10. Kwabi-Addo B, Giri D, Schmidt K, et al. Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:11563-11568.
11. Chapman EJ, Harnden P, Chambers P, Johnston C, Knowles MA. Comprehensive analysis of CDKN2A status in microdissected urothelial cell carcinoma reveals potential haploinsufficiency, a high frequency of homozygous co-deletion and associations with clinical phenotype. Clinical Cancer Research. 2005;11:5740-5747.
12. Ohnishi H, Kawamura M, Ida K, et al. Homozygous deletions of p16/MTS1 gene are frequent but mutations are infrequent in childhood T-cell acute lymphoblastic leukemia. Blood. 1995;86:1269-1275.
13. Bertin R, Acquaviva C, Mirebeau D, Guidal-Giroux C, Vilmer E, Cave H. CDKN2A, CDKN2B, and MTAP gene dosage permits precise characterization of mono- and bi-allelic 9p21 deletions in childhood acute lymphoblastic leukemia. Genes Chromosomes and Cancer. 2003;37:44-57.
14. Iravani M, Dhat R, Price CM. Methylation of the multi tumor suppressor gene-2 (MTS2, CDKN1, p15INK4B) in childhood acute lymphoblastic leukemia. Oncogene. 1997;15:2609-2614.
15. Wong IH, Ng MH, Huang DP, Lee JC. Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications. Blood. 2000;95:1942-1949.
16. Gutierrez MI, Siraj AK, Bhargava M, et al. Concurrent methylation of multiple genes in childhood ALL: Correlation with phenotype and molecular subgroup. Leukemia. 2003;17:1845-1850.
17. Chim CS, Wong AS, Kwong YL. Epigenetic inactivation of INK4/CDK/RB cell cycle pathway in acute leukemias. Annals of Hematology. 2003;82:738-742.
18. Roman-Gomez J, Jimenez-Velasco A, Castillejo JA, et al. Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. Blood. 2004;104:2492-2498.
19. Matsushita C, Yang Y, Takeuchi S, et al. Aberrant methylation in promoter-associated CpG islands of multiple genes in relapsed childhood acute lymphoblastic leukemia. Oncol Rep. 2004;12:97-99.

20. Lemos JA, Defavery R, Scrideli CA, Tone LG. Analysis of p16 gene mutations and deletions in childhood acute lymphoblastic leukemias. Sao Paulo Med J. 2003;121:58-62.
21. Kawamura M, Ohnishi H, Guo SX, et al. Alterations of the p53, p21, p16, p15 and RAS genes in childhood T-cell acute lymphoblastic leukemia. Leukemia Research. 1999;23:115-126.
22. Takeuchi S, Bartram CR, Seriu T, et al. Analysis of a family of cyclin-dependent kinase inhibitors: p15/MTS2/INK4B, p16/MTS1/INK4A, and p18 genes in acute lymphoblastic leukemia of childhood. Blood. 1995;86:755-760.
23. Rasool O, Heyman M, Brandter LB, et al. p15ink4B and p16ink4 gene inactivation in acute lymphocytic leukemia. Blood. 1995;85:3431-3436.
24. Quesnel B, Preudhomme C, Philippe N, et al. p16 gene homozygous deletions in acute lymphoblastic leukemia. Blood. 1995;85:657-663.
25. Harrison CJ, Martineau M, Secker-Walker LM. The Leukaemia Research Fund / United Kingdom Cancer Cytogenetics Group karyotype database in acute lymphoblastic leukaemia: A valuable resource for patient management. British Journal of Haematology. 2001;113:3-10.
26. Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science. 1994;264:436-440.
27. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:9821-9826.
28. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res. 2005;65:6071-6079.
29. Strefford JC, van Delft FW, Robinson HM, et al. Complex genomic alterations and gene expression in acute lymphoblastic leukemia with intrachromosomal amplification of chromosome 21. Proc NatlAcadSciUSA. 2006;103:8167-8172.
30. Barrett MT, Scheffer A, Ben-Dor A, et al. Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. Proceedings of the National Academy of Sciences. 2004;101:17765-17770.
31. Strefford JC, Worley H, Barber K, et al. Genome complexity in acute lymphoblastic leukemia is revealed by array-based comparative genomic hybridization. Oncogene. 2007;26:4306-4318.
32. Jalali GR, An Q, Konn ZJ, et al. Disruption of ETV6 in intron 2 results in upregulatory and insertional events in childhood acute lymphoblastic leukaemia. Leukemia. 2007;22:114-123.
33. Akasaka T, Balasas T, Russell LJ, et al. Five members of the CEBP transcription factor family are targeted by recurrent IGH translocations in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Blood. 2007;109:3451-3461.
34. Metaxa-Mariatou V, Papadopoulos S, Papadopoulou E, et al. Molecular analysis of GISTs: evaluation of sequencing and dHPLC. DNA Cell Biol. 2004;23:777-782.
35. Yarbrough WG, Buckmire RA, Bessho M, Liu ET. Biologic and biochemical analyses of p16(INK4a) mutations from primary tumors. J Natl Cancer Inst. 1999;91:1569-1574.
36. Sakano S, Berggren P, Kumar R, et al. Clinical course of bladder neoplasms and single nucleotide polymorphisms in the CDKN2A gene. International Journal of Cancer. 2003;104:98-103.
37. Schneider-Stock R, Boltze C, Lasota J, et al. High prognostic value of p16INK4 alterations in gastrointestinal stromal tumors. Journal of Clinical Oncology. 2003;21:1688-1697.
38. Deligezer U, Erten N, Akisik EE, Dalay N. Methylation of the INK4A/ARF locus in blood mononuclear cells. Annals of Hematology. 2006;85:102-107.
39. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446:758-764.

40. Usvasalo A, Savola S, Räty R, et al. CDKN2A deletions in acute lymphoblastic leukemia of adolescents and young adults--An array CGH study. Leukemia Research. 2008;32:1228-1235.
41. Kawamata N, Ogawa S, Zimmermann M, et al. Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray. Blood. 2008;111:776-784.
42. Mirebeau D, Acquaviva C, Suciu S, et al. The prognostic significance of CDKN2A, CDKN2B and MTAP inactivation in B-lineage acute lymphoblastic leukemia of childhood. Results of the EORTC studies 58881 and 58951. Haematologica. 2006;91:881-885.
43. Russell LJ, Akasaka T, Majid A, et al. t(6;14)(p22;q32): a new recurrent IGH@ translocation involving ID4 in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Blood. 2008;111:387-391.
44. Teh MT, Blaydon D, Chaplin T, et al. Genomewide single nucleotide polymorphism microarray mapping in basal cell carcinomas unveils uniparental disomy as a key somatic event. Cancer Res. 2005;65:8597-8603.
45. Hu N, Wang C, Hu Y, et al. Genome-wide association study in esophageal cancer using GeneChip mapping 10K array. Cancer Res. 2005;65:2542-2546.
46. Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Res. 2005;65:375-378.
47. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365:1054-1061.
48. Fitzgibbon J, Smith LL, Raghavan M, et al. Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. Cancer Res. 2005;65:9152-9154.
49. Moorman AV, Richards SM, Martineau M, et al. Outcome heterogeneity in childhood high-hyperdiploid acute lymphoblastic leukemia. Blood. 2003;102:2756-2762.
50. Carter TL, Watt PM, Kumar R, et al. Hemizygous p16(INK4A) deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse. Blood. 2001;97:572-574.
51. Ramakers-van Woerden NL, Pieters R, Slater RM, et al. In vitro drug resistance and prognostic impact of p16INK4A/P15INK4B deletions in childhood T-cell acute lymphoblastic leukaemia. British Journal of Haematology. 2001;112:680-690.
52. Dalle JH, Fournier M, Nelken B, et al. p16INK4a immunocytochemical analysis is an independent prognostic factor in childhood acute lymphoblastic leukemia. Blood. 2002;99:2620-2623.
53. Zhou M, Gu L, Yeager AM, Findley HW. Incidence and clinical significance of CDKN2/MTS1/P16ink4A and MTS2/P15ink4B gene deletions in childhood acute lymphoblastic leukemia. Pediatr Hematol Oncol. 1997;14:141-150.
54. Fizzotti M, Cimino G, Pisegna S, et al. Detection of homozygous deletions of the cyclin-dependent kinase 4 inhibitor (p16) gene in acute lymphoblastic leukemia and association with adverse prognostic features. Blood. 1995;85:2685-2690.
55. van Zutven LJ, van Drunen E, de Bont JM, et al. CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia. Leukemia. 2005;19:1281-1284.
56. Graf Einsiedel H, Taube T, Hartmann R, et al. Deletion analysis of p16INKa and p15INKb in relapsed childhood acute lymphoblastic leukemia. Blood. 2002;99:4629-4631.
57. Heerema NA, Sather HN, Sensel MG, et al. Association of Chromosome Arm 9p Abnormalities With Adverse Risk in Childhood Acute Lymphoblastic Leukemia: A Report From the Children's Cancer Group. Blood. 1999;94:1537-1544.
58. Hann I, Vora A, Harrison G, et al. Determinants of outcome after intensified therapy of childhood lymphoblastic leukaemia: results from Medical Research Council United Kingdom acute lymphoblastic leukaemia XI protocol. British Journal of Haematology. 2001;113:103-114.

Table 1: Number of patient samples tested by each technique and at each time-point.

Technique	Number of patients	Number of samples at …		
		Diagnosis	Relapse	Both time-points
Mutation Screening	47	22	26	1
Methylation Status	99	73	29	3
SNP array	98	86	20	8
Array CGH	105	103	3	1
FISH	1,171	1,130	77	36

Abbreviations: SNP - Single nucleotide polymorphism; CGH - comparative genomic hybridisation; FISH - Fluorescence in situ hybridization.

Table 2: Incidence of *CDKN2A* deletions by sex, age, white cell count and cytogenetic subgroup in childhood acute lymphoblastic leukaemia (ALL)

Subgroup	BCP-ALL Cohort			T-ALL Cohort			
	Total	Deletion n (%)	Biallelic deletion n (% of deleted cases)		Total	Deletion n (%)	Biallelic deletion n (% of deleted cases)
Total	864	180 (21%)	78 (43%)	**Total**	266	134 (50%)	83 (62%)
Sex				**Sex**			
Male	473	96 (20%)	43 (45%)	Male	191	96 (50%)	63 (66%)
Female	391	84 (21%)	35 (42%)	Female	75	38 (51%)	20 (53%)
Age Groups				**Age Groups**			
<10 years	679	129 (19%)*	53 (41%)	<10 years	141	81 (57%)*	47 (58%)
10+ years	185	51 (28%)*	25 (49%)	10+ years	125	53 (42%)*	36 (68%)
White Cell Count [1]				**White Cell Count** [1]			
$<50x10^9$/L	636	109 (17%)**	50 (46%)	$<50x10^9$/L	82	37 (45%)	23 (62%)
$>50x10^9$/L	154	55 (36%)**	19 (35%)	$>50x10^9$/L	172	88 (51%)	55 (63%)
Cytogenetic Subgroups [2]				**Cytogenetic Subgroups** [2]			
High hyperdiploidy [3]	279	30 (11%)**	14 (47%)	*TAL1* rearrangements [6]	49	21 (43%)	12 (57%)
ETV6-RUNX1	218	33 (15%)*	15 (45%)	*TLX3* rearrangements [7]	41	31 (76%)**	18 (58%)
t(1;19)(q23;p13)	25	10 (40%)*	2 (20%)	*LMO2* rearrangements [8]	23	9 (39%)	6 (67%)
MLL translocations [4]	22	2 (9%)	1 (50%)	*MLL* translocations [4]	11	2 (18%)	1 (50%)
t(9;22)(q34;q11)	19	11 (58%)**	1 (9%)*	*TLX1* abnormalities [9]	9	8 (89%)*	4 (50%)
iAMP21 [5]	19	5 (26%)	3 (60%)	*AF10-CALM*	9	4 (44%)	1 (25%)
Normal karyotype [10]	52	6 (12%)	6 (100%)**	Normal karyotype [10]	55	23 (42%)	18 (78%)

Key: * $p<0.05$; ** $p<0.01$

Notes: **(1)** Data missing for 86 patients; **(2)** Patients with each specific abnormality were compared with either all other patients with successful cytogenetics or those who tested negative for that particular abnormality by FISH, as appropriate; **(3)** Karyotypes with 51 to 65 chromosomes; **(4)** Patients positive by FISH or with an established translocation by G-banded analysis; **(5)** Intrachromosomal amplification of chromosome 21; **(6)** Involvement of *TAL1* was confirmed by FISH in all cases. Group comprised *SIL-TAL1* (n=43), t(1;14)(p32;q11)/*TAL1-TRA@/TRD@* (n=5) and t(1;14)(p32;q32)/*TAL1-IGH@* (n=1); **(7)** Involvement of *TLX3* (*HOX11L2*) was confirmed by FISH in all cases. In 33 cases *BCL11B* was also confirmed to be involved - i.e. t(5;14)(q35;q32); **(8)** Involvement of *LMO2* was confirmed by FISH in all cases and the partner gene was identified in 20 cases: t(11;14)(p13;q11)/*LMO2-TRA@/TRD@* (n=15), t(7;11)(q34;p13)/*LMO2-TRB@* (n=3), t(7;11)(p15;p13)/*LMO2-TRG@* (n=1) and t(11;14)(p13;q32)/*LMO2-BCL11B* (n=1); **(9)** Involvement of *TLX1* (*HOX11*) was confirmed by FISH in all cases and the partner gene was identified in 7 cases: t(10;14)(q24;q11)/*TLX1-TRA@/TRD@* (n=4) and t(7;10)(q36;q24)/*TLX1-TRB@* (n=3); **(10)** A normal karyotype was defined as the presence of 20 or more normal metaphases and the absence of any cytogenetically visible abnormality or any abnormality detected by FISH.

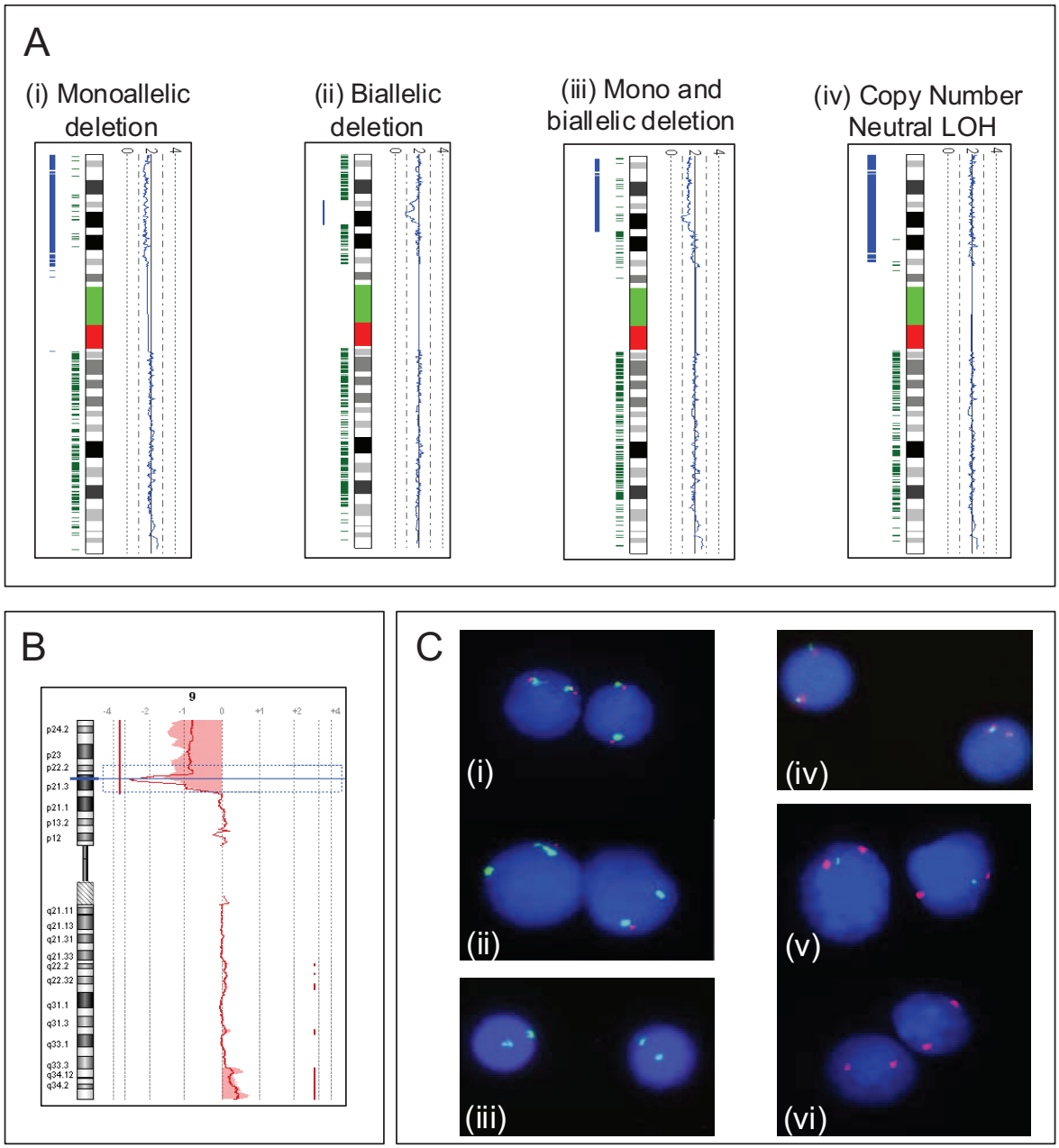

Figure 1: Genomic mapping of chromosome arm 9p: (A) Profiles generated using Copy Number Analyser for Affymetrix® GeneChip (CNAG) software. Ideograms of chromosome 9 are positioned vertically and flanked by a blue copy number plot to the right, which follows 2.0 for normal copy number. To the left of each ideogram heterozygous SNP calls are shown in green and the likelihood of LOH is represented by the thickness of the blue bar. Thus the four profiles show a patient with (i) a monoallelic terminal deletion of 9p; (ii) an interstitial biallelic deletion; (iii) both a terminal monoallelic and interstitial biallelic deletion; and (iv) copy number neutral LOH. **(B)** An array CGH profile for chromosome 9. The chromosome ideogram is positioned vertically with the red line following a log ratio of 0.0 for normal copy number. This profile shows a patient with a terminal monoallelic and interstitial biallelic deletion. The shaded areas define regions of copy number change according to a z-score algorithm. **(C)** FISH analysis: (i-iii) Vysis p16(9p21) (red) / CEP9 (green) probe showing a (i) normal pattern, (ii) monoallelic deletion, or (iii) biallelic deletion. (iv-vi) WRGL CDKN2A (green) / Centromere 9 (red) deletion probe showing (iv) normal pattern, (v) monoallelic deletion, or (vi) biallelic deletion.

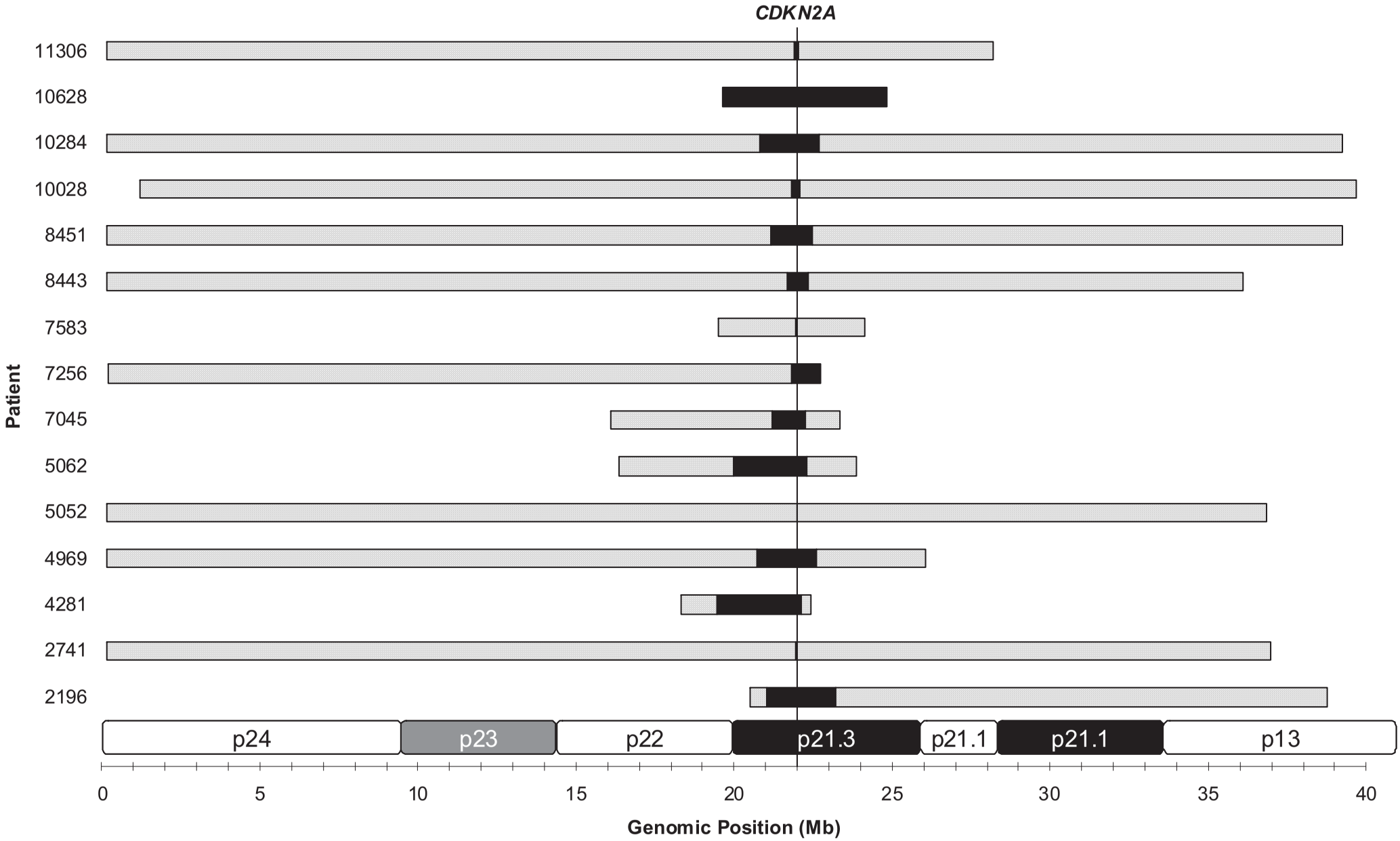

Figure 2: Diagrammatic representation of 15 biallelic deletions as assessed by array CGH analysis. The size of the monoallelic deletion is indicated by the shaded bars and the size of the biallelic deletion by the black bar. The genomic location of *CDKN2A* is shown by the vertical line. A partial ideogram of chromosome 9p is shown at the bottom for reference.