

## ORIGINAL ARTICLE

## Absence of leukaemic fusion gene transcripts in preterm infants exposed to diagnostic x rays

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**Background:** Childhood leukaemias express novel, clonotypic fusion genes that may already be present at birth before the clinical manifestation of leukaemia. Exposure of the fetus to diagnostic x rays is reported to increase the risk of childhood leukaemia, and may do so by generating leukaemic fusion genes. Advances in neonatal medicine in the past decade that have extended the limits of viability of preterm babies down to 23 weeks of gestation have resulted in the increased use of diagnostic x rays to monitor neonatal progress.

**Aim:** To investigate whether exposure of very preterm infants to diagnostic x rays in the neonatal period leads to the development of leukaemic fusion genes.

**Methods:** Peripheral blood samples were collected at birth from very preterm infants (23–30 weeks gestation) and following exposure to diagnostic x rays at intervals of two weeks, until discharge. Cord blood samples from normal full term infants served as controls. Total RNA was extracted from the blood and the expression of the fusion genes *TEL-AML1*, *MLL-AF4*, and *BCR-ABL*, characteristic of three subtypes of childhood leukaemia, was investigated in the preterm and full term infant samples using a nested reverse transcriptase polymerase chain reaction method. Serial pre- and post-x ray samples from 42 preterm babies, pre-x ray samples from an additional 46 preterm infants, and cord blood samples from 100 normal full term infants were screened for fusion gene transcripts.

**Results:** No leukaemic fusion gene transcripts were detected in preterm infants following exposure to diagnostic x rays. A *BCR-ABL* transcript was identified in a single preterm infant prior to x ray exposure. *TEL-AML1* transcripts were detected in cord blood samples from two full term infants. *MLL-AF4* transcripts were not detected in any of the pre- or full term infants tested.

**Conclusions:** Exposure of the preterm infants to x rays in this small series and at the doses used for diagnostic purposes did not induce leukaemic fusion gene expression, but we cannot exclude the possibility that a small proportion of preterm infants may be unusually sensitive to x rays.

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The increased risk of childhood leukaemia following exposure of the fetus to obstetric x rays was first documented in 1956 by Stewart and colleagues,<sup>1</sup> as part of the Oxford Survey of Childhood Cancer (OSCC). Among 269 cases of childhood leukaemia and an equal number of controls, they found that 15.6% of children with leukaemia compared with only 8.9% of control children had previously been exposed to x rays in utero. Although Stewart and Kneale<sup>2</sup> suggested that the risk was related to the radiation dose, these findings were initially greeted with scepticism. However, they were subsequently confirmed by others,<sup>3</sup> by reanalysis of the OSCC data<sup>4</sup> and in meta-analyses.<sup>5</sup> The considerable reduction in the use of x rays during pregnancy that these results have brought about<sup>6</sup> has made an important contribution to reducing the risk of childhood cancer and leukaemia arising from medical intervention.<sup>7</sup>

Developments in fetal and neonatal medicine in recent years have enabled an increasing proportion of babies born as early as 22–23 weeks gestation to survive.<sup>8–10</sup> However, the need to monitor neonatal progress, including respiratory disorders, means that very preterm infants often undergo a considerable number of x ray examinations.<sup>11–15</sup> While dose estimates suggest that the excess number of cancers owing to neonatal x rays is extremely low, and far outweighed by the benefits in terms of neonatal fetal survival,<sup>10 13 15 16</sup> increasing radiation exposure at a relatively early gestational stage is a matter of concern.

Uncertainties remain about exposing very preterm infants to radiation for the following reasons. (1) No long term follow up studies of cancer risk in relation to preterm birth have been published. (2) There appears to be no lower threshold dose for x rays below which cancer does not constitute a risk. (3) Pre-

term babies may differ from those born at term with respect to their x ray sensitivity. (4) It has recently been shown that the novel fusion genes characteristic of certain childhood leukaemias can occur in normal infants.

Recent evidence<sup>17</sup> suggests that chromosomal double strand breaks (DSBs), in some cases caused by ionising radiation, may lead to the acquired chromosomal translocations associated with the generation of the novel expressed hybrid or fusion genes that are typical of leukaemia.<sup>18–20</sup> Studies of monozygotic twins who both developed leukaemia, in some cases at different ages, have revealed identical clonotypic fusion gene sequences in both twins that suggest that these arise before birth as a single event in one twin, and are transmitted to the other twin through the conjoined placental circulations.<sup>21 22</sup> Further evidence that fusion genes can arise prenatally comes from studies in which fusion genes have been backtracked to birth in children who later develop leukaemia.<sup>23</sup>

Leukaemic fusion genes provide potentially informative biomarkers of leukaemic cell populations, and the development of these genes as a consequence of the mis-repair of DSBs induced by diagnostic x rays could provide an important measure of leukaemia risk associated with medical x ray exposure. To investigate this specifically in preterm babies exposed to

**Abbreviations:** ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; DAP, dose area product; DSB, double strand break; ERR, excess relative risk; FFD, focus to film distance; NMU, neonatal medical unit; OSCC, Oxford Survey of Childhood Cancer; RT-PCR, reverse transcriptase polymerase chain reaction

diagnostic *x* rays, we have used a sensitive nested reverse transcriptase polymerase chain reaction (RT-PCR) to amplify fusion genes known to be associated with childhood leukaemias. Here, we describe our results using this assay to detect three types of fusion gene transcripts: *TEL-AML1*, generated by the fusion of the *TEL(ETV6)* gene on chromosome 12 with the *AML1 (CBFA2)* gene on chromosome 21, and present in ~20–25% of childhood precursor B cell acute lymphoblastic leukaemia (ALL)<sup>24</sup>; *MLL-AF4*, generated by the fusion of the *MLL* gene on chromosome 11 with the *AF4* gene on chromosome 4, and found in at least 70% of infant ALL and acute myeloid leukaemia (AML)<sup>25</sup>; and *BCR-ABL*, generated by the fusion of the *BCR* gene on chromosome 9 with the *ABL* gene on chromosome 22 and found in under 5% of childhood ALLs.<sup>26</sup>

## METHODS

### Study population

The study was carried out on preterm babies born at 23–30 weeks gestation who were admitted to the Neonatal Medical Unit (NMU) at St Mary's Hospital, Manchester between 1998 and 2000. The study had ethical approval from the Central Manchester & Manchester Children's University Hospitals NHS Trust local research ethics committee. Blood samples (~0.5 ml) from preterm infants and clinical data were obtained with informed maternal consent. A total of 88 preterm infants born between 23 and 30 weeks of gestation were included in the study. Blood samples were collected in sterile 5 ml bottles containing 1 ml of cell culture medium (RPMI 1640 with Glutamax 1 and 25 mM HEPES, Life Technologies Ltd, Paisley, UK) and 30 µl EDTA, as soon after birth as possible, and prior to *x* ray exposure, and then subsequently at two weekly intervals until discharge. Cord blood samples obtained from an anonymised series of normal full term infants served as non *x* rayed controls.

Clinical details of the preterm babies and their mothers were obtained from obstetric and paediatric case notes, and included data on obstetric conditions, birth details, and resuscitation. Neonatal information included the need for ventilator support, oxygen dependence, and details of drugs administered and blood transfusions.

### *x* Ray dosimetry

All of the exposures were undertaken using a Hitachi Sirius 125B mobile *x* ray unit located on the NMU. The date, time, type of examination, exposure factors, and the area of the baby exposed to *x* rays to the nearest mm<sup>2</sup> were measured for each radiograph. The *x* ray output of the machine was measured using a calibrated dose meter over a range of clinical exposure factors. The dose area product (DAP) was calculated from the appropriate output, corrected for focus to film distance (FFD), *x* ray transmission of incubator lid and exposed area of each film. Since radiation risk is normally quantified by "effective dose", correction factors to convert DAP to effective dose have been published by the National Radiological Protection Board (NRPB).<sup>27</sup> However, appropriate risk data are not available for calculating this dose in preterm babies ≤1500 g. As an alternative, the "energy imparted" to the infant was used as a measure of radiation risk. This was calculated as follows: energy imparted (mJ) = dose in air at FFD × area of field (cm<sup>2</sup>) × conversion factor.<sup>14</sup> Mean absorbed doses received by individual infants were subsequently calculated in relation to body weight.

### Cell lines

Reference cell lines derived from leukaemias expressing the relevant fusion genes were used as positive controls, and were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany ([http://](http://www.dsmz.de/)

[www.dsmz.de/](http://www.dsmz.de/)). These were as follows: REH<sup>28</sup> expressing *TEL-AML1*; MV4;11<sup>29</sup> expressing *MLL-AF4*; and BV173<sup>30</sup> expressing *BCR-ABL*. An Epstein-Barr virus transformed normal lymphoblastoid cell line (SV18), prepared from a normal donor, and not expressing the above fusion genes, was used as a negative control. All cell lines were cultured in RPMI-1640 containing 10% fetal calf serum.

### RNA extraction

Total RNA was extracted from whole blood samples and from the fusion gene positive and negative reference cell lines using RNeasy Standard and Blood Mini Kits (Qiagen Ltd, Crawley, UK). The RNA was eluted in 40 µl of RNase free water; 1 µl of this was diluted 1/50 and used to measure the Optical Density<sub>260/280</sub> in order to obtain the yield, concentration, and purity of the RNA. To avoid cross contamination, all RNA extractions were carried out in a separate laboratory using dedicated pipettes and aerosol resistant tips.

### Amplification of fusion gene transcripts by RT-PCR

Leukaemic fusion gene transcripts were amplified using a nested reverse transcriptase (RT) polymerase chain reaction (PCR) method. A 1 µg aliquot of total RNA was amplified using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Amersham, UK). Reactions were carried out in 0.2 ml tubes, each containing a lyophilised bead comprising the reagents required for performing a single tube RT-PCR reaction. To each tube 1 µg total RNA, 1.5 µl oligo(dT)<sub>12–18</sub> primer (0.5 µg/µl), and 6 µl of first round PCR primers (5 µM each)<sup>21 24 32 33</sup> (see table 1) were added, and the volume brought to 60 µl with RNase free water. Reaction mixtures were vortexed to fully dissolve the beads, centrifuged, and incubated at 42°C for one hour to carry out cDNA synthesis. First round PCR was then performed in the same tubes on a PCR Express thermocycler (Hybaid, Middlesex, UK) using the following conditions: 3 minutes at 94°C, 32 cycles of 94°C for 30 seconds, 66°C for 30 seconds (*TEL-AML1*), 64°C (*MLL-AF4*) or 58°C (*BCR-ABL*), 72°C for 30 seconds, and finally 72°C for 3 minutes. A 2 µl aliquot of the RT-PCR products were then subjected to second round amplification using internal primers<sup>21 24 32 33</sup> (table 1) as follows: 2 µl 10 × PCR buffer (200 mM Tris HCl, 500 mM KCl, pH 9.0), 2 µl primer mix (5 µM each), 3 µl dNTPs (2 mM each), 1.5 µl MgCl<sub>2</sub> (25 mM), 1 µl W1 (1%), 0.1 µl *Taq* DNA polymerase (5 U/µl) (Gibco, Life Technologies Ltd, UK), 8.4 µl H<sub>2</sub>O, and 2 µl first round PCR product. Second round cycling conditions were: 94°C for 3 minutes, 38 cycles of 94°C for 15 seconds, 55°C for 15 seconds, 72°C for 15 seconds, and a final step at 72°C for 3 minutes. Fusion of the *BCR* and *ABL* genes may give rise to several hybrid transcripts owing to alternative splicing.<sup>34</sup> In order to detect commonly expressed *BCR-ABL* transcripts two pairs of first and second round primers were used in multiplex reactions.<sup>33</sup> As a control for RNA integrity separate PCR amplifications were carried out on each sample to detect β actin expression. All second round PCRs were carried out in five replicates. To prevent cross contamination separate areas were used to set up PCRs, to carry out amplifications, and to analyse the PCR products. Dedicated pipettes with aerosol resistant tips were used to prepare PCR mixtures. Distilled water was used as a negative control for PCR.

### Detection of fusion gene transcripts

To detect PCR amplification, 5 µl of each PCR product was electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light (Biorad Gel Doc 2000, BioRad UK, Hemel Hempstead). The remaining PCR product was dot blotted onto positively charged nylon membranes (Hybond-N+, Pharmacia,

**Table 1** First and second round fusion gene primers and breakpoint specific SSO probes

	5'-3' sequence	Specificity	Location	Ref
<i>Primer</i>				
TELS841	ATC ATG CAC CCT CTG ATC CT	<i>TEL-AML1</i>	5' external	21
AMLAS765	TGG GCA GGG TCT TGT TGC AG	<i>TEL-AML1</i>	3' external	21
B12	CGT GGA TTT CAA ACA GTC CA	<i>TEL-AML1</i>	5' internal	24
AM3	CTC GCT CAT CTT GCC TGG	<i>TEL-AML1</i>	3' internal	24
MLLAF4A	AGA GCA GAG CAA ACA GAA	<i>MLL-AF4</i>	5' external	32
MLLAF4B	GCT GAG AAT TTG AGT GAG	<i>MLL-AF4</i>	3' external	32
MLLAF4C	AAG TGG CTC CCC CGC CCA AGT AT	<i>MLL-AF4</i>	5' internal	32
MLLAF4D	TTG GGT TAC AGA ACT GAC ATG	<i>MLL-AF4</i>	3' internal	32
BCRA	CGG GAG CAG CAG AAG AAG TC	<i>BCR-ABL b2a2 /b3a2</i>	5' external	33
BCRC	TTG TCG TGT CCG AGG CCA CC	<i>BCR-ABL e1a2</i>	5' external	33
ABLA	TGT GAT TAT AGC CTA AGA CCC GGA G	<i>BCR-ABL b2a2 /b3a2/e1a2</i>	3' external	33
BCRB	GTG AAA CTC CAG ACT GTC CAC AGC A	<i>BCR-ABL b2a2 /b3a2</i>	5' internal	33
BCRD	CAA GAC CGG GCA GAT CTG GCC C	<i>BCR-ABL e1a2</i>	5' internal	33
ABLB	TCC ACT GGC CAC AAA ATC ATA CAG T	<i>BCR-ABL b2a2 /b3a2/e1a2</i>	3' internal	33
β-ACTIN 5'	TGA AGT CTG ACG TGG ACA TC	β-ACTIN 5'	5'	
β-ACTIN 3'	ACT CGT CAT ACT CCT GCT TG	β-ACTIN 3'	3'	
<i>Probe</i>				
TEL-AML1 P1	AGA ATA GCA GAA TGC ATA CTT	<i>Probe 267bp transcript TEL-AML1</i>		31
TEL-AML1 P2	GAA TAG CAG ATG CCA GCA C	<i>Probe 228bp transcript TEL AML1</i>		31
AF4 P	TAG GGA AAG GAA ACT TGG ATG	<i>Probe MLL-AF4</i>		32
b2a2 P	GCT GAA GGG CTT CTT CCT TAT TGA TG	<i>Probe b2a2 transcript BCR-ABL</i>		33
b3a2 P	GCT GAA GGG CTT TTG AAC TCT GCT TA	<i>Probe b3a2 transcript BCR-ABL</i>		33
e1a2 P	GCT GAA GGG CTT CTG CGT CTC CAT	<i>Probe e1a2 transcript BCR-ABL</i>		33

Amersham, UK) using a vacuum manifold. The PCR products were denatured in situ with 0.5 M NaOH, 1.5 M NaCl followed by 1.5 M NaCl, 0.5 M Tris HCl (pH 7.4), and the membranes baked at 80°C for two hours to fix the DNA. The fixed PCR products were then hybridised with 10 pmol of a translocation junction specific oligonucleotide probes labelled with 10 µCi  $\gamma$ -<sup>32</sup>P-adenosine triphosphate (ATP) by T4 polynucleotide kinase (Promega, Southampton, UK).<sup>31-33</sup> Hybridisation was carried out at 58°C overnight, and the membranes washed briefly at room temperature in 2×SSC/0.1% SDS followed by a second wash with tetramethyl ammonium chloride (TMAC) solution (3.0 M TMAC, 50 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.1% SDS) at 58°C for 15 minutes. The membranes were sealed between plastic film, and autoradiography, to detect probe hybridisation, was carried out in real time on an InstantImager (Canberra Packard, Berkshire, UK).

#### Sensitivity of fusion gene transcript detection

Experiments were carried out to determine the sensitivity of the nested RT-PCR fusion gene assay. RNA (1 µg) samples from the leukaemic reference cell lines carrying *TEL-AML1*, *MLL-AF4*, or *BCR-ABL* were serially diluted ( $10^{-1}$ – $10^{-6}$ ) with RNA from the fusion gene transcript negative control cell line SV18, and subjected to nested RT-PCR, then probed as described above.

#### Direct sequencing of fusion gene products

Positive PCRs were cycle sequenced using ABI BigDye terminator cycle sequencing reactions (version 2). Briefly, 8 µl of Terminator Ready Reaction mix (Applied Biosystems, Warrington, UK) was added to 3.2 pmol of each primer, 5 ng of purified PCR product, and deionised water in a volume of 20 µl, and the reactions amplified on a PCR Express thermal cycler (Hybaid, Middlesex, UK) as follows: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Extension products were purified by precipitation with 95% ethanol and 3 M sodium acetate (pH 4.6), pelleted, and washed with 70% ethanol. Sample pellets were resuspended in Template Suppression Reagent (Applied Biosystems, Warrington, UK) and electrophoresed on an ABI 310 Prism Genetic Analyser (Applied Biosystems).

## RESULTS

### Study group

We obtained blood samples from 88 very preterm infants born between 23 and 30 weeks gestational age. The clinical characteristics of these infants and their mothers were similar to a larger series of 230 preterm infants (which included the sampled group), born between 23 and 30 weeks during the same period (table 2). This suggests that the sampled group was truly representative of the preterm infants on the NMU over the period of the study. We also obtained serial blood samples from 42 of the 88 preterm infants after they had been exposed to diagnostic x rays. Post x ray samples could not be obtained from about half of these infants (n = 46) because they were either transferred back to their referring hospital or died within the first two weeks postnatally from prematurity or other causes.

### x Ray dosimetry

Table 3 shows details of x rays in relation to postnatal age in the group of 42 preterm infants serially blood sampled after x ray exposure. In the first two weeks postpartum these infants had 1–19 x ray episodes, the mean number/infant being 6.9. The number of x rays received by the total sampled series of 42 preterm infants while on the NMU was 483, the maximum number of x ray episodes for an individual baby being 43 over an eight week period. Mean absorbed doses received by infants in the first two weeks ranged from 0.002 to 0.13 mGy, the maximum mean absorbed dose received by an individual infant being 0.32 mGy over an eight week period.

### Detection of fusion gene expression

Since we were not certain whether cells carrying leukaemic fusion gene transcripts would be present in preterm infants, either before or after x ray exposure, we used a sensitive nested RT-PCR assay to detect small numbers of fusion gene expressing cells. Tests on RNA from the reference leukaemia cell lines (REH, MV4;11, and BV173) diluted with normal RNA (SV18) showed that our method amplified leukaemic fusion genes with sensitivity thresholds of  $10^{-6}$  for *TEL-AML1*,  $10^{-5}$  for *MLL-AF4*, and  $10^{-4}$  for *BCR-ABL* (fig 1).

**Table 2** Characteristics of preterm infants tested for fusion genes

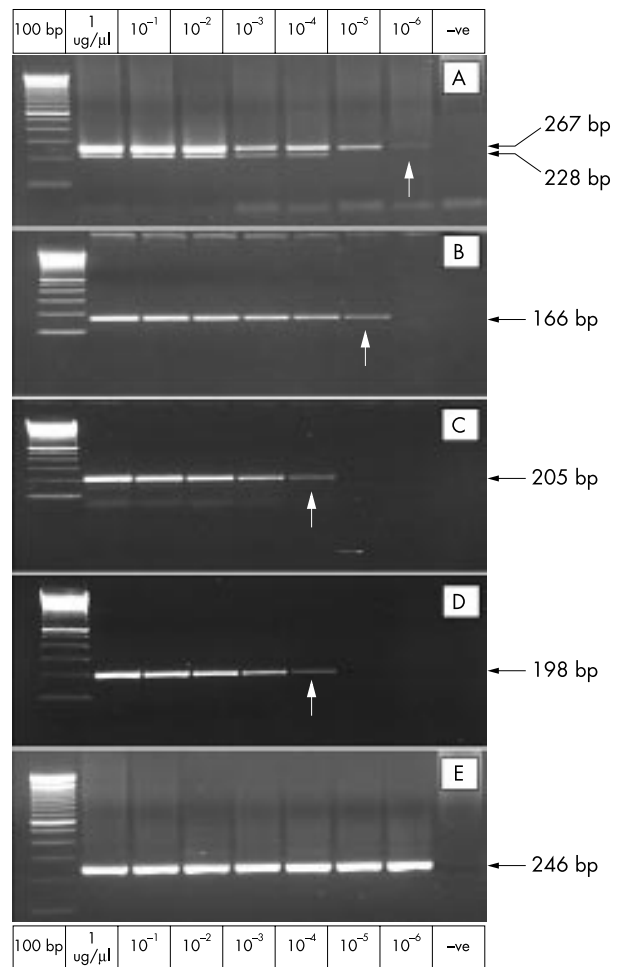
Infant and maternal characteristics	Fusion gene tested n=88		Total infant series n=230	
	n	%	n	%
<b>Infants</b>				
Male	45	51	119	52
Female	43	49	111	48
<b>Birth weight (g)</b>				
<750	25	28	66	29
751–1250	46	52	114	49.5
>1251	17	19	50	21.5
<b>Gestational age (wk)</b>				
≤25	21	24	55	24
26–28	36	40	81	35
≥29	31	35	94	41
<b>Ethnicity</b>				
Caucasian	73	83	182	79
Asian	7	8	31	13.5
Afro-Caribbean	2	2	7	3
Oriental	3	3.5	3	1.5
Other	3	3.5	7	3
<b>Mothers</b>				
<b>Parity</b>				
Primiparous	30	34	59	25.5
Multiparous	58	66	171	74.5
Singleton birth	62	70.5	170	74
Multiple births	26	29.5	60	26
Pregnancy induced hypertension	22	25	56	24
PROM	25	28	63	27
<b>Delivery</b>				
Vaginal	48	54	118	51
LSCS	40	46	112	49
<b>O<sub>2</sub> dependence</b>				
28 days	31/40	77.5	75/120	62.5
36 weeks	13/23	56.5	37/86	43
PDA	28	32	50	22
NEC	11	12.5	27	12
ROP	5	6	8	3.5
Sepsis	32	36	94	41
Mortality	18	20.5	52	22.5

PROM, premature rupture of fetal membranes; LSCS, lower segment caesarean section; PDA, patent ductus arteriosus; NEC, necrotising enterocolitis; ROP, retinopathy of prematurity.

### Analysis of preterm and control blood samples for fusion genes

Blood samples from the total series of 88 preterm infants were tested for the expression of the three leukaemic fusion genes (*TEL-AML1*, *MLL-AF4*, and *BCR-ABL*) by nested RT-PCR. These samples included 46 preterm infants obtained at birth, and serial samples from 42 preterm infants obtained both before and at two weekly intervals after *x* ray exposure. The serial samples from the 42 infants totalled 136 (42 pre-*x* ray, 94 post-*x* ray), the number of post-*x* ray samples ranging from one to four per infant (table 4).

We did not detect any of the three types of leukaemic fusion gene transcripts in the preterm infant samples following *x* ray

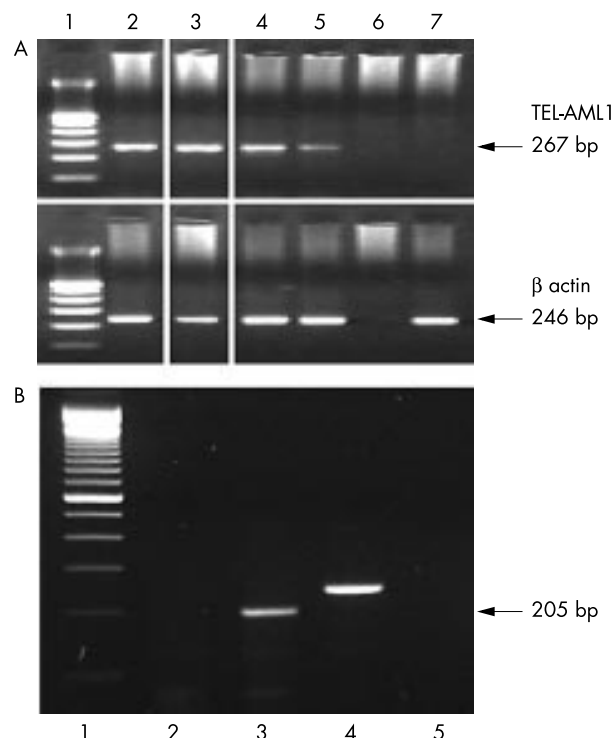


**Figure 1** RT-PCR sensitivity experiments. (A) *TEL-AML1* (REH). (B) *MLL-AF4* (MV4;11). (C) *BCR-ABL* minor breakpoint junction (BV173). (D) *BCR-ABL* major breakpoint junction (BV173). (E)  $\beta$  actin positive control (REH). Arrows indicate sensitivity threshold.

exposure. However, we detected a *BCR-ABL* transcript in one preterm infant (fig 2) prior to *x* ray exposure. This infant was one of a pair of identical twins (twin 1) born at 27 weeks gestation. Discordant amniotic fluid volumes indicated that the twins exhibited fetofetal transfusion syndrome and amniotic fluid reduction was carried out two weeks prior to delivery. At birth twin 1 (the donor) had oligohydramnios, and weighed 971 g. A haemoglobin of 97 g/l necessitated five blood transfusions, the first of which preceded blood sampling for fusion gene transcript analysis by 24 hours. Twin 1 was reviewed after about four months and was making excellent progress. Twin 2 (the recipient) had a birth weight of 1176 g and a

**Table 3** Numbers of *x* rays and absorbed doses received by preterm babies in relation to postnatal age

	Postnatal age (weeks)			
	2	4	6	8
Number of babies	42	30	17	5
Number of <i>x</i> rays—range	1–19	4–27	4–31	8–43
Number of <i>x</i> rays—mean	6.9	11.2	16.5	26.8
Total number of <i>x</i> rays	292	338	280	134
Mean absorbed dose (mJ/kg)—range	0.002–0.13	0.017–0.21	0.02–0.24	0.07–0.32
Mean absorbed dose (mJ/kg)—mean	0.072	0.087	0.130	0.188



**Figure 2** (A) Second round TEL-AML1 transcript showing positive full term infant cord blood samples. Lane 1, 100 bp ladder; 2, positive cord blood (a); 3, positive cord blood (b); 4,  $10^{-4}$  REH (TEL-AML1+) cells diluted in SV18 (normal lymphoid) cells; 5, as 4, but REH diluted  $10^{-5}$  with SV18; 6, water (negative) control; 7, normal lymphoid cell line SV18. (B) Second round BCR-ABL transcript from single preterm infant sample (for details, see text). Lane 1, 100 bp ladder; 2, b2a2/b3a2 (p210) transcript; 3, E1a2 (p190) transcript; 4, β actin positive control; 5, water (negative) control.

haemoglobin of 191 g/l. Unfortunately no blood sample for fusion gene analysis was available from twin 2.

In the cord blood samples from 100 normal full term non x rayed control infants, we detected *TEL-AML1* fusion gene expression in two infants (fig 2).

### Sequencing of fusion gene transcripts

RNA preparations from the three infants (one preterm, two full term) expressing fusion gene transcripts were reamplified in RT-PCR and cycle sequenced to determine the nucleotide sequences of the breakpoint junctions, in comparison with the same sequences in the Genbank database. The *BCR-ABL* transcript in the preterm infant (twin 1) had the same sequence as the *BCR-ABL* minor breakpoint sequence coding the 190 kD (p190) minor breakpoint peptide of the BCR/ABL protein (GenBank accession number AF113911). Both of the *TEL-AML1* transcripts from the full term infants included the 39 bp exon 2 of the *AML1* gene commonly expressed by this fusion gene (GenBank accession number S78496).

### DISCUSSION

It is known that exposure of the fetus to x rays in utero increases the risk of childhood leukaemia by about 40%,<sup>1-7</sup> and it has been suggested that there is no lower threshold dose below which there is no risk from prenatal radiation exposure.<sup>7</sup> Since very preterm babies born between 24 and 30 weeks are the equivalent of second/third trimester fetuses, exposure to x rays in the immediate postnatal period might be expected to pose a similar risk to exposure in utero, though the radiation doses used for preterm babies are lower than those that were associated with an increase risk of leukaemia following in utero exposure. Fletcher and colleagues<sup>11</sup> estimated that at an average radiation dose to the chest or abdomen, neonates received about 0.07 mGy, sufficient to cause only about two extra cases of childhood cancer each year in the UK. Sutton and colleagues<sup>15</sup> quantified the exposure of 55 very low birthweight neonates (mean gestational age 28.8 weeks) who received a total of 498 radiographs and three radiological procedures (median five per infant), and found a mean surface entrance dose of 33.1 μGy and a mean absorbed dose of 0.04 mGy. This contrasts with the 42 very preterm infants babies serially blood sampled after x ray exposure in our study who were exposed to 1–19 x ray procedures in the first two weeks postpartum, the mean number per infant being 6.9 with a maximum absorbed dose of 0.32 mGy over an eight week period, compared with 54 mGy over 30 weeks reported by Sutton and colleagues.<sup>15</sup> Unlike radiation doses delivered for typical x ray examinations in adults where there has been progress towards dose standardisation,<sup>35</sup> no such national standards exist for preterm x rays. This may explain why the average effective dose imparted to infants under 2 years old undergoing head computed tomography scans reported in one study was six times higher than that for adults.<sup>36</sup>

In vitro studies have shown that exposure of cell lines to radiation, albeit at high doses, can lead to the generation of leukaemic fusion genes.<sup>37, 38</sup> Although the risk of developing leukaemia from the x ray doses used in preterm babies is extremely low,<sup>10, 13, 16</sup> the absence of a lower dose threshold does not rule out a biological effect. Stochastic events resulting from radiation induced DNA damage may be misrepaired, leading to the chromosome translocations and the generation of leukaemic fusion genes that characterise childhood leukaemias. Although the physical effects of radiation exposure and their biological consequences, in terms of DNA breakage and consequent instability have been studied in detail,<sup>39</sup> the long term effects of single (α) particles on lymphocytes have only been studied relatively recently.<sup>40</sup> Even though it is not yet possible to predict the effects of a single x ray track delivered to the haemopoietic cells of a preterm baby, recent evidence suggests that it is the proximity of the partner genes involved in translocations that may be an important factor in their development.<sup>41</sup> In the case of the ABL and BCR genes, irradiation of lymphocytes alters chromatin structure, leading to shortening of the distances between these genes, thereby increasing the probability of translocation.<sup>42</sup>

**Table 4** Number of blood samples and tests for leukaemic fusion genes

Group	Infants		Number of tests			
	Number	Samples	TEL-AML1	MLL-AF4	BCR-ABL	Total
Preterm	88	182	910	910	910	2730
pre-x ray	46	46	230	230	230	690
pre- and post-x ray	42	136	680	680	680	2040
Full term	100	100	500	500	500	1500

Evidence that certain leukaemic fusion genes can occur prenatally<sup>23–43</sup> means that they are a potentially important biomarker of incipient leukaemia.<sup>22</sup> However, the detection of fusion genes such as *BCR-ABL* in normal adults<sup>44–45</sup> and *TEL-AML1* in children<sup>46–48</sup> suggests that other mutations are required for the development of overt leukaemia. We assumed that exposure to *x* rays might result in the generation of fusion genes in preterm infants, but it is possible that *x* rays might increase their frequency and the probability of causing additional mutations, in a dose dependent manner. We used a nested RT-PCR technique that is capable in our hands of detecting 0.001–0.0001% fusion gene positive cells among a large excess of cells not expressing fusion genes. This level of sensitivity was required since we had no prior indication of the number of fusion gene positive cells that were present. We screened for *TEL-AML1*, *MLL-AF4*, and *BCR-ABL* transcripts as these are among the commonest fusion genes in childhood leukaemia. Since most of the infants in our study were at the limits of viability, we were only able to obtain about 0.5 ml of blood from each infant, and the number available for serial study was limited by early mortality and other clinical considerations. Although we detected no fusion gene transcripts in any of the post-*x* ray samples, this does not rule out the possibility that *x* rays may have caused other biological effects. Moreover, we tested samples obtained over a period of 2–8 weeks after *x* rays, which may have been insufficient time to detect the long term effects of genomic instability on the development of fusion genes.<sup>49</sup> We detected a fusion gene transcript (*BCR-ABL*) in only one preterm baby, and we strongly suspect that this originated from transfused blood. Bearing in mind the limitations of our sampling method, and that we only tested peripheral blood, our results suggest that any fusion genes that do arise during gestation may occur at a later stage. This contrasts with the findings of Uckun and colleagues<sup>32</sup> who detected *MLL-AF4* transcripts in 4/16 fetal bone marrow and 5/13 fetal liver samples from 15–22 week abortuses. Our failure to detect *MLL-AF4* transcripts in preterm and normal full term infants agrees with the findings of Trka and colleagues<sup>48–50</sup> and Kim-Rouille and colleagues,<sup>51</sup> and contrast with evidence of a high frequency of partial tandem duplications in the *MLL* gene in normal donors.<sup>52</sup>

Although our results are reassuring in suggesting that *x* ray exposure of preterm babies may not lead to the development of leukaemic fusion genes, this conclusion needs to be treated with caution. Leukaemic fusion genes clearly do arise in utero in a small percentage of normal infants as shown in this and other studies. The argument that fusion genes are not induced by fetal exposure to radiation seems untenable if dose extrapolation from epidemiological studies suggesting “that there is no threshold dose below which no effect is produced”<sup>7</sup> is correct. The explanation may therefore be that leukaemic fusion genes are not generated in sufficient numbers or in the appropriate target cells by the *x* ray doses currently used in preterm babies. The annual dose of background radiation results in the delivery of about 1 mGy of  $\gamma$  rays to an 8  $\mu$ m nucleus.<sup>53</sup> In comparison, the maximum absorbed dose that we observed was 0.32 mGy over an eight week period. If continued for a year, this would roughly double the annual dose received by preterm babies. If we take the radiation risk coefficient for childhood leukaemia as being 2.5%/Gy,<sup>7</sup> this would lead to an individual probability of developing radiogenic leukaemia from preterm *x* ray examination of about 1 in a million. So while the molecular effects of *x* ray exposure may not be negligible, radiation risk estimates suggest that the risk to preterm babies is minute. Looked at another way, although the risk of childhood leukaemia is approximately 1 in 1760 under 15 years of age, the risk of a radiogenic leukaemia is only 1 in 4400 if the excess relative risk (ERR) owing to fetal *x* ray

exposure is 40%. Since the *x* ray doses to which preterm babies are exposed are about two orders of magnitude less than this, we would expect the ERR to be similarly reduced.

The caveat to this reasoning is that these calculations are based on the assumption that the risk of leukaemia in preterm infants following *x* ray exposure is the same as that in full term infants. Although there is a paucity of data on this point, there is evidence from clonogenic assays to suggest that cord blood lymphocytes are more radiosensitive than adult lymphocytes,<sup>54</sup> and it would seem reasonable to expect that this sensitivity might be even greater at a fetal age of 24–30 weeks gestation. Furthermore, the fourfold higher frequency of congenital anomalies in preterm babies compared to those at term,<sup>55</sup> and the suggestion that about one in 20 antenatal events may lead to malformation and cancer in childhood,<sup>56</sup> suggests that certain preterm babies might be constitutionally more vulnerable than term babies to the damaging effects of *x* rays, though this clearly requires further investigation. Finally, the possibility that a small proportion (<2%) of preterm infants may be genetically hypersensitive to low dose *x* rays cannot be excluded on the basis of the results presented in this paper.

We may also have failed to detect fusion gene transcripts owing to the limited amount of preterm infant blood available for analysis. The average amount of RNA that we obtained per ~0.5 ml sample was 6.9  $\mu$ g, corresponding to about  $7 \times 10^5$  cells. Our sample thus represented about 0.5% of the circulating blood volume of an average preterm infant born at <30 weeks, and we used about 1  $\mu$ g RNA to test for each of the three fusion genes, corresponding to ~ $10^5$  cells<sup>52</sup> per gene. This means that we analysed slightly less than 0.1% of the circulating blood in each infant, with a detection threshold of 1–10 pg per fusion gene. To ensure that all of the material in each 1  $\mu$ g RNA sample was tested for each fusion gene, we divided all of the cDNA synthesised in the first round RT-PCR into five replicates for the second round PCR, and tested each of these for fusion gene transcripts.

The cumulative incidence rate for all types of leukaemia in children under 15 years in the UK is 1 in 1760, and for childhood ALL it is 1 in 2183.<sup>37</sup> Since *TEL-AML1* is thought to be present in about 25% of childhood ALL,<sup>31</sup> equivalent to about 1 in 8000 of the total childhood population, our result showing that it is present in ~2% of normal full term infants suggests that this fusion gene may be present at a frequency ~160 times greater than in overt leukaemia. This suggests that *TEL-AML1* gene fusion may not give rise to leukaemia unless it involves the appropriate cell lineage and stage of differentiation, and that additional mutations are required for overt leukaemia to develop. It is also possible that immune surveillance mechanisms are able to eliminate *TEL-AML1* positive cells<sup>58–59</sup> in the majority of infants. This would suggest that certain types of childhood leukaemia may arise as a result of defective immune surveillance, and that the immunosuppressive effects of *x* rays may pose a greater risk than their capacity to cause DNA damage.

In summary, we have been unable to detect three types of leukaemic fusion gene transcript associated with childhood ALL in preterm infants born between 24 and 30 weeks and exposed to multiple diagnostic *x* rays in the postpartum period. We suggest that currently used *x* ray doses only constitute a very small risk of causing leukaemia if radiation risk estimates assume that preterm infants are not significantly more radiation sensitive. However, based on the limited numbers of preterm babies that we were able to study we cannot rule out the possibility that the factors that shorten gestation also render the fetus more susceptible to radiation, and that the genetic background of the fetus might have some part to play in this. The presence of a *BCR-ABL* transcript in one preterm infant was not owing to *x* ray exposure, but could be explained by a previous blood transfusion. Since the frequency

of *TEL-AML1* transcripts in normal infants greatly exceeds the frequency of childhood ALL with this fusion gene, there may be natural mechanisms that protect against the progression of fusion transcript positive cells to leukaemia.

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## Absence of leukaemic fusion gene transcripts in preterm infants exposed to diagnostic x rays

P F Ravetto, R Agarwal, M L Chiswick, et al.

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