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Original Paper

Screening for Neuroblastoma in Late Infancy by Use of EIA (Enzyme-linked Immunoassay) Method: 115 000 Screened Infants in Austria

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The aim of this study was to investigate the feasibility of a neuroblastoma screening programme for children in late infancy, based on collaboration of general paediatricians and practitioners in Austria, using the technique of enzyme-linked immunoassay (EIA) for biochemical analyses. Analysis of catecholamine metabolites in spot urine samples by EIA with high performance liquid chromatography as a backup was undertaken. Austrian infants (median age 8.7 months) were screened. Overall compliance was 30%. The EIA method had a high rate (6.7%) of false-positive results. 28 infants were admitted to hospital. In 15 cases, neuroblastoma was found (four stage 1, five stage 2B, six stage 3). The EIA method can be used for neuroblastoma screening, but requires a backup analytical technique in order to avoid unnecessary hospital admissions. The stage distribution and biological features of neuroblastomas diagnosed by screening at a later age are different from those detected by earlier screening. Screening in late infancy might be of more benefit than early screening.

Key words: neuroblastoma screening, urine catecholamines, EIA, high performance liquid chromatography, biological features, N-myc

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INTRODUCTION

FOLLOWING APPARENTLY favourable reports from Japan of mass screening for neuroblastoma in 6-month-old infants [1–4], a screening programme was introduced in Austria in 1991 [5]. However, at that time first reports of the risk of overdiagnosis by early screening appeared and it was argued that the declining mortality from neuroblastoma in Japan was unlikely to be a consequence of the mass screening programme introduced there in 1985 [6–11]. Some argued that screening at 6 months of age detects predominantly cases which would otherwise regress spontaneously. The reports concluded that screening at an age of 6 months could not be generally recommended [8, 10]. Because of this, in the Austrian project, screening was postponed into late infancy. Furthermore, a new EIA method [12] was used for analysis of the catecholamine metabolites vanillylmandelic acid (VMA) and homovanillic acid (HVA) in spot urine samples.

The aim of the Austrian study was to examine the efficacy of the EIA method for mass screening for neuroblastoma and to evaluate the possible effect of screening at a later age on the stage distribution and biological features of neuroblastoma cases diagnosed by screening.

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PATIENTS AND METHODS

Collection of urine samples on filter paper

In January 1991, general paediatricians and practitioners of Austria, which has a birth rate of 94 000 infants per year, were invited to participate in the neuroblastoma screening programme and distribute filter papers for spot urine collec-

tion. The filter strips were handed out to patients of 7–9-month-old infants at a routine checkup. Instructions were printed on the filter paper to inform parents about the aim of the test and how to collect urine. Parents were advised to insert the filter strip into the napkin for urine collections, to dry it well afterwards and then send it directly to the laboratory.

Analysis of urine catecholamines

Urine was eluted from filter paper by phosphate buffer base solution and quantitative assessment of VMA and HVA was made using a new EIA method (Yamasa Shoyu Co., Choshi, Japan [12]). Creatinine was determined by the Jaffe reaction. The analysis was fully automated by use of a Beckman work station. For photometric assessment, a Eurogenetics plate reader connected to a data station was used. Cut-off values were defined as mean value +2.5 standard deviations calculated from the results of 500 samples collected from healthy infants of the relevant age range. Apparent false-positive results, especially for HVA, most likely caused by cross-reactions, were excluded from calculations of cut-off values which were 26 μg/mg creatinine for VMA and 30 μg/mg creatinine for HVA.

All positive results from the EIA method were confirmed by high performance liquid chromatography (HPLC, Biorad, Richmond, California, U.S.A.). HPLC cut-off values were determined similarly and were 20 μg/mg creatinine for VMA and 37 μg/mg creatinine for HVA.

Furthermore, every 200th sample was analysed by both EIA and HPLC methods so that the results of the two methods could be compared by paired *t*-test and the correlation coefficient calculated. Clear false-positive outliers (VMA >40 μg/mg creatinine, HVA >50 μg/mg creatinine by EIA but a negative result by HPLC) were excluded from the calculations.

Retests amd hospital admission

Following an inadequate urine specimen (low urine content, stool contamination, insufficiently dried filter paper), parents were asked by letter to repeat the test. When values of either HVA or VMA were elevated by both EIA and HPLC methods, a double retest was requested. Parents were asked to collect one spot urine sample in the morning and one in the evening. Parents were advised to avoid banana or vanilla in baby's food at least one day prior to urine collection.

When retest results were elevated, parents were informed of the results by phone and letter and the infant was admitted to the local children's hospital. The parents were given a letter addressed to the paediatrician of the local hospital asking for further investigations consisting of abdomen ultrasonography, chest X-ray, serum analysis (including LDH, NSE and ferritin) and a 24-h urine collection.

Clinical examination and treatment

If a neuroblastoma was found by the clinical investigation, treatment was initiated by the local hospital and in most cases this was according to the Austrian treatment protocol for neuroblastoma patients [13]. Primary resection of the tumour was attempted and where this was not possible, it was recommended that a biopsy be performed. In some cases, where the tumour was not totally resectable, no chemotherapy was given in accordance with a recent SIOP trial [14]. This practice was employed only for cases with favourable biological markers in anticipation of spontaneous regression or maturation of the residual tumour [15].

If no tumour was found by sonography and X-ray, further investigations were made dependent on laboratory findings. If 24-h urine catecholamines and NSE were in the normal range, no further investigations were performed. When urine catecholamines remained high and/or where serum NSE (neurone-specific enolase) levels were elevated further investigations were performed (computerised tomography and/or MIBG (radioactive iodine metaiodobenzoguanidine) scintigraphy and/or bone marrow puncture). If these investigations also failed to reveal a tumour, the case was classified as 'false-positive'.

Staging and analysis of tumour material

Each neuroblastoma was staged according to both the Evans' and the INSS classification [16, 17]. Histological tumour classification according to Shimada and Joshi [18, 19] was performed in 11/15 cases.

Biological features of the tumours were assessed by several techniques. Tumour samples were resuspended in RPMI 1640 plus antibiotics and 10% fetal calf serum. R-banding was performed by employing a chromomycin/distamycin/DAPI staining technique [20]. Double fluorescence *in situ* hybridisation (FISH) analyses were carried out on cytospin slides prepared from uncultured tumour cells using pUC1-77 (D1Z1) specific for the centromeric region of chromosome 1 and the VNTR probe p1-79 (D1Z2) specific for the subtelomeric region of 1p (1p36.33). Hybridisation conditions and detection of the hybridised probes were performed as reported previously [21]. Ploidy of tumour cells was determined by flow cytometry according to standard conditions using a FACStar flow cytometer (Becton Dickinson). N-MYC amplification was analysed from the resected tumours using FISH and Southern blot analysis.

Follow-up of true-positive and false-positive cases, search for false-negative cases

Follow-up of neuroblastoma patients was performed every 3 months by contacting the hospitals where the children were treated.

In false-positive cases, the screening test was repeated every 2 months until the levels of urinary catecholamines metabolites returned to normal. The national neuroblastoma registry was then checked to find out if any of these children subsequently developed clinical neuroblastoma.

In order to ascertain false-negative cases (children with a negative screening result who later presented clinically with neuroblastoma), data on cases registered with the Austrian neuroblastoma registry were compared with the screening register.

RESULTS

Compliance

Between January 1991 and July 1995, 115 448 urine samples from infants aged 7–12 months were received by the laboratory. There were 311 132 children eligible for screening during this time who were not screened (Figure 1). Compliance increased slowly from 9% in 1991 and has been stable at around 30% since 1992. However, regional

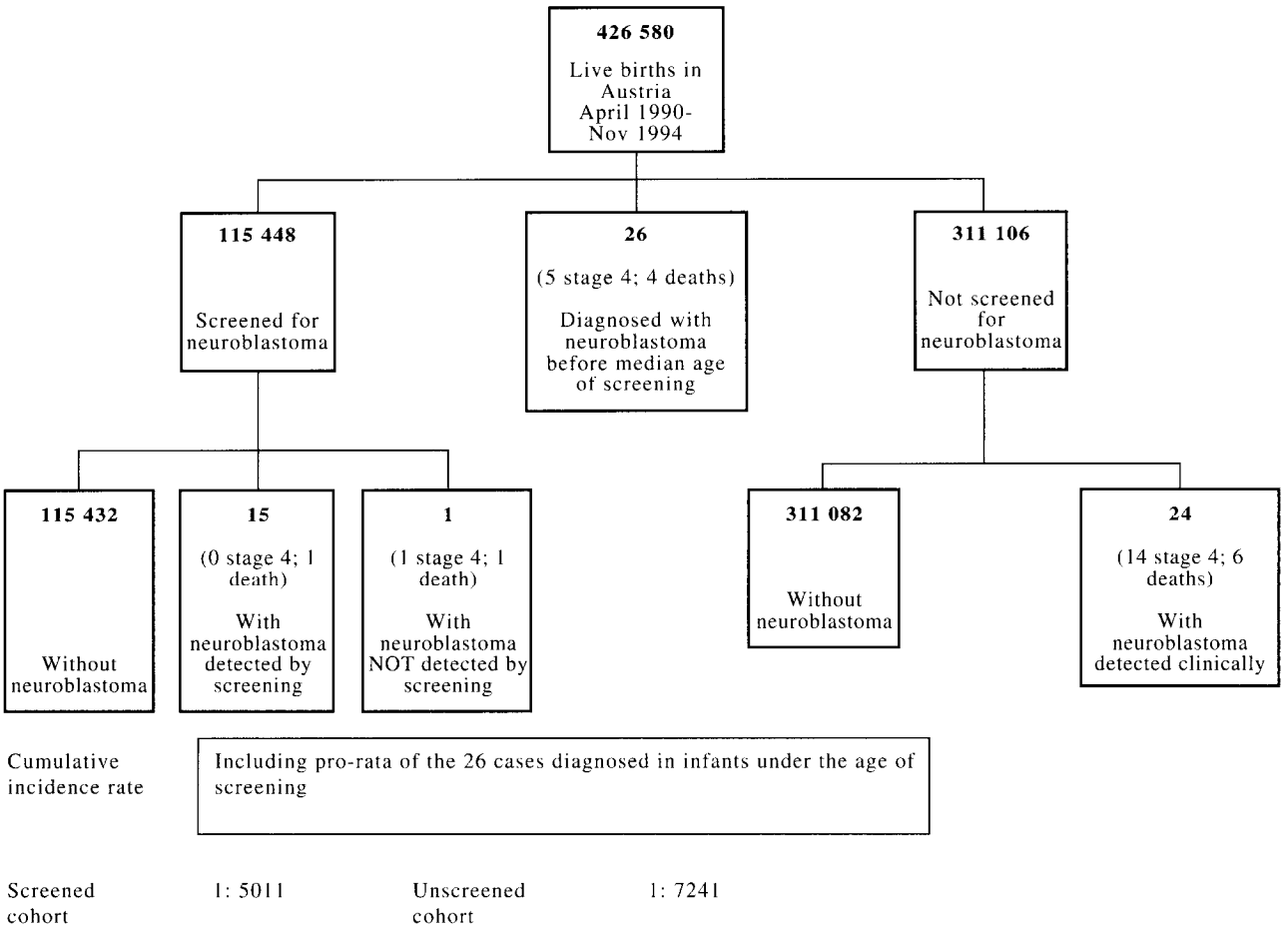

Figure 1. Neuroblastoma in the Austrian birth cohort April 1990–November 1994.

compliance rates were very different, ranging between 12% and 73%. These differences were mainly a consequence of differing acceptance of the screening programme by the local health boards.

Comparison of EIA and HPLC method

Every 200th sample was analysed by both EIA and HPLC methods. The results for these 562 samples are shown in Figures 2 and 3.

EIA results for VMA were significantly higher than results of HPLC (EIA mean VMA 14.2 μg/mg creatinine S.D. 5.12; HPLC mean 8.90 μg/mg S.D. 4.95 paired *t*-test $P < 0.0000$), though the results were correlated ($r = 0.39$; $P < 0.0000$). For HVA, the higher results were obtained by HPLC (HPLC mean HVA 18.82 μg/mg, S.D. 7.72; EIA mean HVA 16.22 μg/mg S.D. 6.9), again these differences were significant (paired *t*-test, $P < 0.0000$), though they were correlated ($r = 0.43$; $P < 0.0000$).

The EIA and HPLC results of patients diagnosed by screening are shown in Table 1. There was significant correlation between tumour volume and catecholamine metabolite levels.

Screening results and frequency of retests (Figure 4)

For 89.1% of samples, the primary EIA analysis was negative. 4.2% of samples were insufficient, most frequently due to low urine content, insufficient drying and stool contamination. In the remaining 6.7% of analyses, EIA results were positive and these were repeated using HPLC (Figure 3), 0.9% of samples were confirmed as positive by HPLC and thus required a double retest. The retest confirmed elevated urine catecholamines in 28 infants (0.025% of all screened infants). These infants were admitted to the local children's hospital.

Clinical examination

In 13/28 screened positive infants, no neuroblastoma was found on clinical investigation. 8 of these infants had normal levels of urine catecholamines under the conditions of clinical urine collection. Apart from abdominal ultrasonography, chest X-ray and laboratory examinations, no further investigations were performed in these children. In the remaining 5 children, urine catecholamine levels remained elevated, and were, in 3 cases, parallelled by elevated serum NSE (>15 ng/ml), but no neuroblastoma was found even by intensified investigations (MIBG scan, bone marrow aspirates and computerised tomography of chest and abdomen). A follow-up by analysis of further urine samples was performed for these infants and showed normalisation of values 2, 4, 5, 10 and 13 months after the first sample collection, respectively.

In 15/28 cases, a neuroblastoma was found. Three of these tumours were not seen in the first ultrasonography. Two of these tumours were detected by a second ultrasound examination, 1 tumour was found only by computerised tomography.

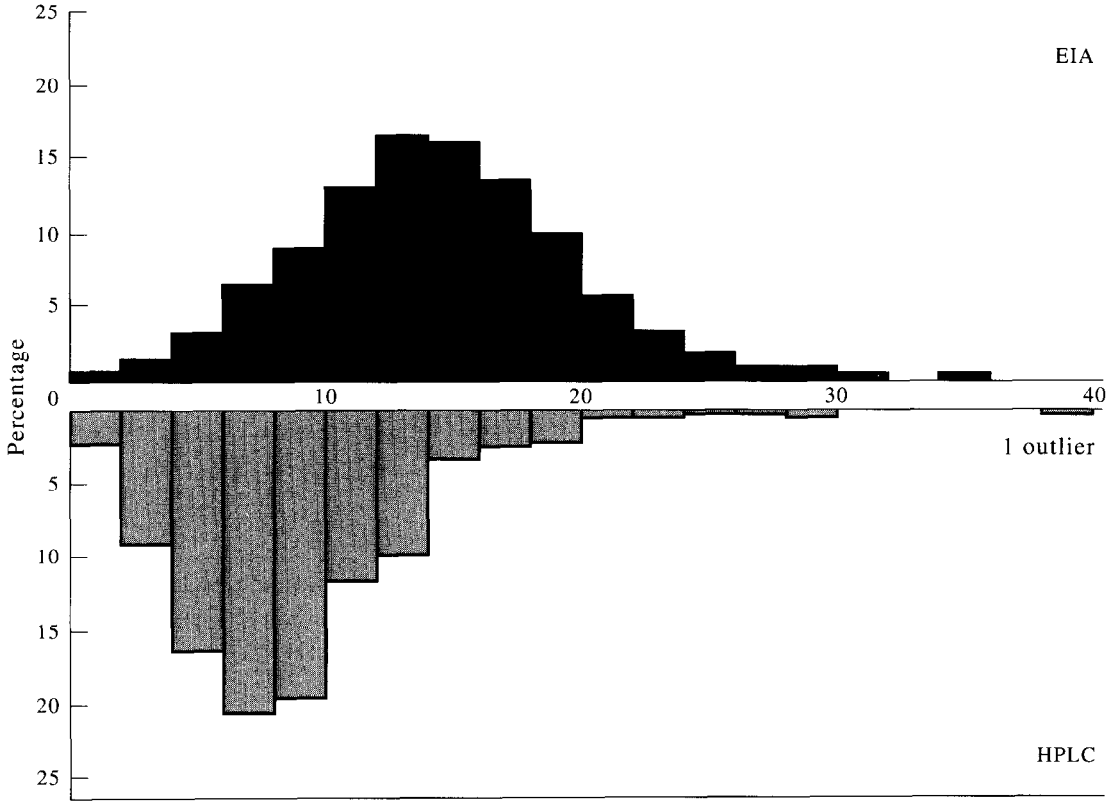

Figure 2. Distribution of VMA results (μg/mg creatinine) of 562 spot urine samples analysed by EIA and HPLC.

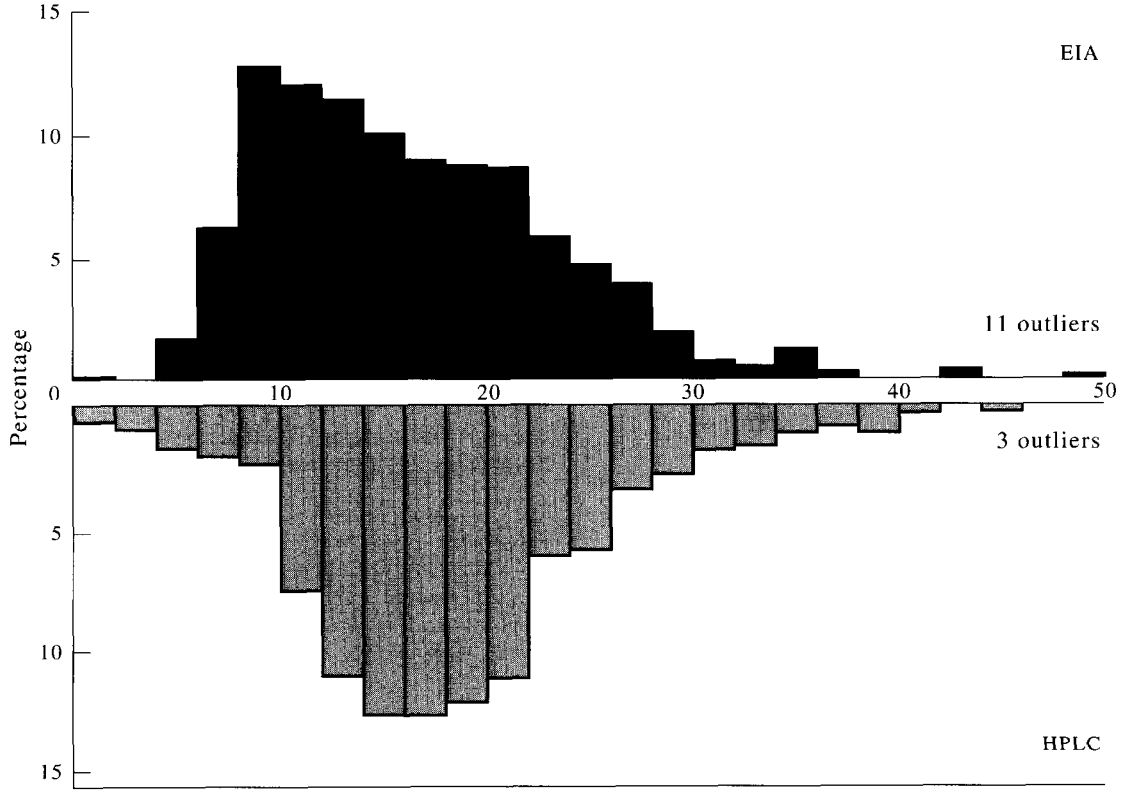

Figure 3. Distribution of HVA results (μg/mg creatinine) of 562 spot urine samples analysed by EIA and HPLC.

Table 1. INSS stage, tumour volume and urine catecholamine values of neuroblastoma patients detected by the Austrian screening programme between January 1991 and July 1995. The cases are sorted in ascending order of tumour volume

Patient	INSS stage	Tumour volume (ml)	EIA VMA/HVA [26/30]*	HPLC VMA/HVA [20/37]*
1	1	14	35/24	32/28
2	3	18	50/31	38/29
3	2B	20	53/49	37/45
4	2B	32	35/39	35/72
5	1	40	48/39	38/36
6	3	45	40/41	29/53
7	1	48	67/93	53/72
8	2B	70	67/33	59/50
9	3	80	145/48	130/67
10	2B	115	88/68	170/139
11	3	125	165/53	147/66
12	1	175	66/55	55/50
13	3	175	168/86	146/106
14	2B	190	117/146	143/123
15	3	280	301/198	278/185
r†			0.84/0.83	0.82/0.81
P-value			0.0001/0.0001	0.0002/0.0002

Correlation between tumour volume and urine catecholamine values was calculated.
*Cut-off values. r† = Pearson's correlation coefficient.

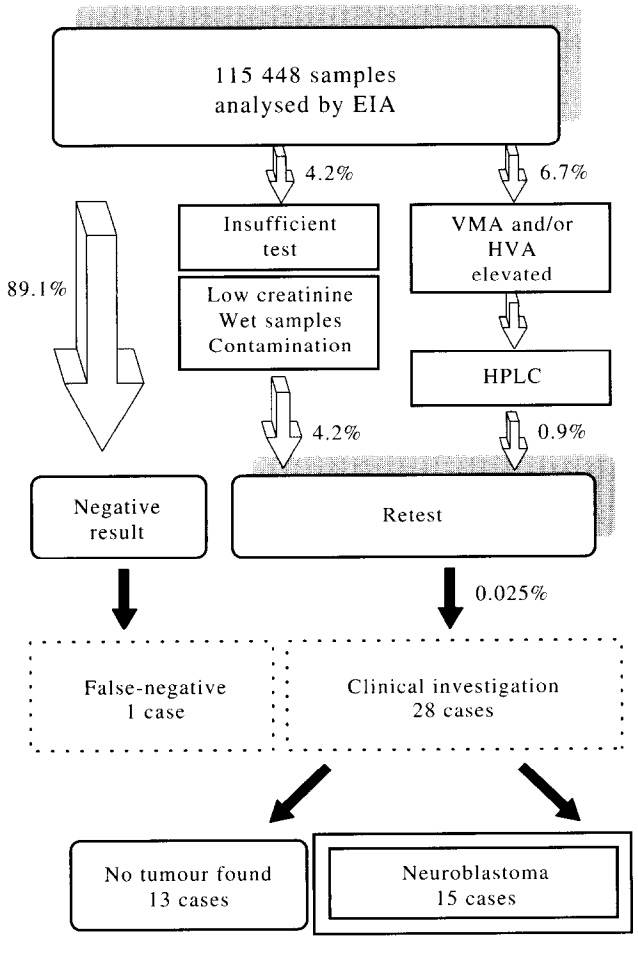

Figure 4. Flowchart of the 115 448 urine samples analysed by the Austrian neuroblastoma screening programme between January 1991 and July 1995.

Primary site and stages

All 15 neuroblastomas were abdominal tumours, and 6 were of adrenal origin. According to the Evans' classification, 3 cases were classified as stage I, 3 were stage II, and 9 were stage III. Table 2 summarises the characteristics of the 15 neuroblastoma cases.

According to the INSS classification, 4 tumours were stage 1, 5 were stage 2B and 6 were stage 3.

Serum analysis

Serum lactate dehydrogenase (LDH) was elevated (>360 U/l) in 8/15 neuroblastoma cases. NSE was elevated (>15 ng/ml) in 14/15 patients. Serum ferritin was >300 ng/ml in 0/15 patients. The HVA/VMA ratio of the HPLC method was >1 in 4/15 patients.

Histological classification and biological features

9 out of 11 tumours had favourable histology using Shimada and Joshi classifications. The remaining two tumours were classified as unfavourable by both systems. One tumour was near-tetraploid and the remaining 13/14 tumours were triploid (two with structural chromosomal aberrations). None of 14 patients investigated for 1p36 deletion had loss of heterozygosity (LOH) for the short arm of chromosome 1. N-myc analysis revealed amplification in 2/14 patients (>5-fold and >10-fold, respectively).

Treatment

11 tumours were considered to be resectable and primary surgical treatment was performed. In 4 of these cases the tumours and in 2 cases the tumour and affected lymph nodes were totally resected and no further therapy was given. One patient received postoperative chemotherapy despite complete resection of the primary tumour. In 3 cases, residual tumour remained, but no chemotherapy was administered in anticipation of spontaneous regression of the residual tumour. The remaining child died during surgery.

Table 2. Characteristics of the 15 neuroblastoma cases detected by the Austrian screening programme between January 1991 and July 1995

Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Age at diagnosis (months)	9.1	8.9	10	11.8	8.8	8.9	9	8.3	10.4	10.3	9.1	9.9	11	9	8.3
Stage (INSS)	III (3)	III (1)	III (3)	III (3)	II (2B)	III (3)	III (2B)	I (1)	III (2B)	III (2B)	II (2B)	II (2B)	I (1)	III (3)	III (3)
VMA/HVA ratio (HPLC)	1.5	1.1	0.5	1.4	0.5	1.3	0.8	0.7	1.1	1.2	1.2	1.2	1.1	2.2	1.9
NSE	136	59	28	37	14	20	45	75	40	34	34	43	35	40	22
Ferritin	82	17	24	6	18	35	66	121	47	12	29	64	21	33	50
LDH	512	482	361	277	278	252	379	465	292	294	334	458	352	439	413
Ploidy-cytogenicity	3n, str.aberr	3n	3n	n.d.	3n	3n	3n	3n	3n, str.aberr	3n	3n	<4n	3n	3n	3n
N-MYC	5–30x	1x	1x	n.d.	1x	1x	1x	10–100x	1x	1x	1x	1x	1x	1x	1x
1p deletion	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no
Shimada	fav.	fav.	fav.	n.d.	fav. (differ.)	n.d.	fav.	unfav.	fav.	fav.	fav.	fav.	unfav.	n.d.	n.d.
Joshi	fav. (grade 2)	fav.	fav.	n.d.	fav. (grade 1)	n.d.	fav.	unfav. (grade 3)	fav. (grade 2)	fav. (grade 2)	fav. (grade 1)		fav. (grade 1)	n.d.	n.d.
Outcome	Dead	NED	NED	VGPR	NED	NED	NED	NED	NED	regress.	NED	rel. (bone, BM)	NED	PR	VGPR
Follow-up (months)	51	35	29	27	25	23	22	19	15	14	14	10	10	9	8

3n, triploidy; 4n, tetraploidy; str. aberr., structural aberrations; fav., favourable; unfav., unfavourable; NED, no evidence of disease; VGPR, very good partial response; n.d., not done; rel., relapse; regress, regression; BM, bone marrow.

In the remaining 4/15 cases, primary resection was impossible due to tumour size and localisation. In these cases, a biopsy was performed in order to gain tumour material for histological and biological analyses. The patients were treated with chemotherapy and 2 had surgical treatment following this.

Follow-up and outcome of patients identified by screening

As of January 1996, the median follow-up time for patients diagnosed by the Austrian neuroblastoma screening programme is 19 months (range 8–51 months). One patient died as a consequence of intra-operative bleeding and haemorrhagic shock during primary surgery. The remaining 14 patients are alive, 9 without evidence of disease, 4 with residual disease under therapy or in expectation of spontaneous regression of the residual tumour. One patient who was stage 2B at initial diagnosis had a disseminated relapse 7 months after complete resection of primary tumour and lymph nodes and is currently receiving chemotherapy.

Follow-up of 'false-positive' cases and search for 'false-negative' cases

None of the 13 children with a 'false-positive' screening result have so far developed clinical neuroblastoma.

A search for false-negative cases was conducted by comparing data of the Austrian neuroblastoma registry with data of the 115 448 screened infants by name and date of birth. Median follow-up time for screened infants is 27 months and the median age of screened infants as of January 1996 is 35.7 months. One 'false-negative' case was found. This child had a normal screening result at the age of 12 months, but presented clinically with stage 4 neuroblastoma and very high urine catecholamine levels at the age of 37 months. This child died in January 1996 after 6 months of treatment.

Neuroblastoma in unscreened children

In the April 1991–July 1995 birth cohort, 26 children were diagnosed clinically with neuroblastoma before they reached the median age of screening. 24 cases of neuroblastoma were diagnosed in the 311 106 children who were not screened (Figure 1).

DISCUSSION

Screening for neuroblastoma was started in Japan more than 20 years ago [1, 28]. The Japanese experience encouraged researchers to set up screening programmes in other countries [5, 22–25]. Several analytical methods have been used and HPLC and gas chromatography–mass spectrometry (GC–MS) have become standard methods for quantitative assessment of urine catecholamines [22, 24–27]. However, the number of samples that can be analysed daily by one machine is limited using these techniques. The EIA method of analysis provides an alternative and has been used in some Japanese screening laboratories since 1993 [28]. This method requires minimal equipment, laboratory space and laboratory staff. It is easy to handle, can be fully automated and enables the user to analyse a very large number of samples daily. However, in our hands there were a high number of false-positive results, especially for HVA, most likely resulting from cross-reactivity. Therefore, at present, the EIA method requires a confirmatory method in

order to avoid unnecessary retests and distress to concerned parents. The price of the commercially available EIA kit is relatively high when compared to the costs of HPLC analysis, and we consider that this technique can only be recommended if the price is reduced and if a backup method (HPLC or GC–MS) is available.

In the Austrian screening programme, the combination of EIA and HPLC methods led to the detection of 15 asymptomatic neuroblastoma cases among 115 448 screened infants.

Compliance varied between regions from 12 to 73%, suggesting that an overall compliance in excess of 70% may be possible with greater encouragement of health boards in some regions. The overall low compliance rate (30%) and the absence of a control group do not allow a reliable epidemiological analysis of the Austrian project. However, some results are different from those of the Japanese and the North American programmes which detected predominantly low stage neuroblastomas with favourable biological features, although some higher stage tumours were found, but overall there was a 2-fold increase in the incidence of the disease [4, 7, 9, 23, 28–33]. Within our target population, some 26 children with neuroblastoma were diagnosed clinically before they reached the screening age. Apportioning these cases to screened and unscreened cohorts on a pro-rate basis (7 to the screened and 19 to the unscreened group), allows estimation of the cumulative incidence. In the screened group, which has a median age of 35.7 months, the cumulative incidence was 1:5011 ((16 + 7)/115 448). This incidence is lower than that reported from the Japanese studies (approximately 1:4000 [28]) and the North American project (1:4160 [32]), although the children in these studies have been followed up for longer, but is higher than the 'natural' cumulative incidence of neuroblastoma in unscreened children up to 15 years of age [34]. It is also appreciably higher than the cumulative rate reported for the first 5 years of life in Germany which is 134 per million (1:7463) [35], which is similar to the cumulative incidence of 1:7241 estimated for the unscreened Austrian group. The cumulative incidence in the screened group is likely to increase further with future diagnosis of neuroblastoma in children whose screening test result was normal (false-negative cases). It is, therefore, probable that a proportion of the screening detected cases in Austria would have regressed spontaneously, but it is possible that this proportion is lower than that in other screening projects in which children were screened at an earlier age.

More advanced stages and several tumours with intermediate or unfavourable biological markers were detected by our programme than in the Japanese reports which describe the detection of relatively few such "unfavourable" cases [36, 37].

Altered stages and biological features may reflect the differences in screening age (6 months in Japan, 3 weeks and 6 months in North America, 7–12 months in Austria).

The notion of 'over diagnosis' in screened populations and the suspicion that neuroblastoma screening at a young age might detect mainly cases which would otherwise regress spontaneously have become the main arguments against this type of screening [7–10, 32, 38].

An optimal screening should (1) detect only cases which are unable to regress spontaneously and, therefore, not lead to an increased incidence, (2) not miss cases which present clinically at a later age, (3) lead to an 'early' diagnosis of the 'bad subset' and consequently improve the outcome, (4) reduce mortality. However, it seems questionable whether these criteria can be fulfilled by a single screening at any age. We believe that, in several of our cases, spontaneous regression was unlikely. If stage 3, unfavourable histology, N-MYC amplification, di- or tetraploidy, elevated LDH and HVA/VMA ratio >1 were considered as independent risk factors, 11/15 patients had at least one risk factor, 3 had two risk factors and 4 had three or more risk factors. Alternatively, the observation of one false-negative case and an increased neuroblastoma incidence implies that our screening has not fully fulfilled the first and second criteria given above.

Recently, some screening programmes have postponed screening to the 12th month of life in order to reduce overdiagnosis and to detect a greater proportion of the 'bad subset' of neuroblastoma [25, 26, 39]. Our above-mentioned false-negative case demonstrates that some cases will not be detected even when screening is delayed until this age.

The experience of the Japanese and North American screening groups imply that screening for neuroblastoma should be postponed until after the 6th month of life. However, the optimal age to screen has still to be found, and may not exist. As an alternative, single screening could be replaced by multiple screening, but only if multiple screening can be performed at a reasonable price, e.g. by a cheaper EIA test than is currently available. However, screening can only be worthwhile, if, as a consequence of early detection of disease, mortality is reduced as lives are saved. No study has yet been able to address this major issue adequately. For this purpose, huge population-based controlled studies are necessary [40] and most likely some more years will pass until a clear answer about the benefit of neuroblastoma screening is available.

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