A practical approach to the clinical diagnosis of Ewing’s sarcoma/primitive neuroectodermal tumour and other small round cell tumours sharing EWS rearrangement using new fluorescence in situ hybridisation probes for EWSR1 on formalin fixed, paraffin wax embedded tissue

U Yamaguchi, T Hasegawa, Y Morimoto, U Tateishi, M Endo, F Nakatani, A Kawai, H Chuman, Y Beppu, M Endo, H Kurotaki, K Furuta

Background: Over 90% of Ewing’s sarcoma/primitive neuroectodermal tumour (ES/PNET) cases have the t(11;22) chromosomal rearrangement, which is also found in other small round cell tumours, including desmoplastic small round cell tumour (DSRCT) and clear cell sarcoma (CCS). Although this rearrangement can be analysed by fluorescence in situ hybridisation (FISH) using routinely formalin fixed, paraffin wax embedded (FFPE) tissues when fresh or frozen tissues are not available, a sensitive and convenient detection method is needed for routine clinical diagnosis.

Aims: To investigate the usefulness of newly developed probes for detecting EWS rearrangement resulting from chromosomal translocations using FISH and FFPE tissue in the clinical diagnosis of ES/PNET, DSRCT, and CCS.

Methods: Sixteen ES/PNETs, six DSRCTs, and six CCSs were studied. Three poorly differentiated synovial sarcomas, three alveolar rhabdomyosarcomas, and three neuroblastomas served as negative controls. Interphase FISH analysis was performed on FFPE tissue sections with a commercially available EWSR1 (22q12) dual colour, breakapart rearrangement probe.

Results: One fused signal and one split signal of orange and green, demonstrating rearrangement of the EWSR1 translocated region, were detected in each of the 39 tumour samples. None of the negative controls demonstrated rearrangement of the EWSR1 region.

Conclusions: Interphase FISH using this newly developed probe is sensitive and specific for detecting the EWS rearrangement on FFPE tissues and is of value in the routine clinical diagnosis of ES/PNET, DSRCT, and CCS.
MATERIALS AND METHODS
Specimen selection
Archival tumour specimens were retrieved from the pathological files at the laboratory of pathology, National Cancer Centre (NCC), Tokyo, Japan. A series of small round cell tumours with morphological and immunophenotypical resemblance diagnosed and treated at the centre was selected for study. Tumours included 16 primary ES/PNETs (10 seen in house and six in consultation), six DSRCTs, and six CCSs. Three alveolar rhabdomyosarcomas, three poorly differentiated synovial sarcomas, and three neuroblastomas served as negative controls because there are no EWS rearrangements in these tumour types. For light microscopic study, all specimens were routinely fixed in 10% formalin and embedded in paraffin wax, and 4 μm thick sections were then cut and stained with haematoxylin and eosin. Frozen tissues were available for reverse transcribed polymerase chain reaction (RT-PCR) analysis in seven of 16 ES/PNETs, five of six CCSs, and in all cases of DSRCT, alveolar rhabdomyosarcoma, and poorly differentiated synovial sarcoma.

We defined ES/PNET as a small round cell tumour; focal rosette-like arrangement of tumour cells was seen in some cases (fig 1A). All tumours were consistently immunopositive for CD99 (O-13). Of 16 ES/PNETs, frozen tissue was available from seven at the time of diagnosis for RT-PCR analysis, and the EWS–FLI1 fusion gene was detected in five. There were five male and 11 female patients, with ages at the time of diagnosis ranging from 11 to 72 years (mean, 39). The sites of involvement were the bone in four patients, kidney in two, duodenum and pancreas in one patient each, and other soft tissue with various anatomical locations in eight patients, two of which occurred in subcutaneous locations (cases 4 and 14) (table 1).

The DSRCTs were classified primarily on the basis of compact nests consisting of small round cells separated by abundant desmoplastic stroma (fig 1B), with an undifferentiated appearance, and had strong immunoreactivity for vimentin, broad spectrum keratin (AE1/3), epithelial membrane antigen, desmin, and neurone specific enolase. In all cases, desmin had a distinctive, intense, intracytoplasmic dot-like. The diagnosis was further confirmed by the presence of the EWS–WT1 fusion gene demonstrated by RT-PCR analysis. Table 2 summarises the clinical findings of the six DSRCTs.

The CCSs were composed of compact nests of rounded or fusiform cells with clear cytoplasm (fig 1C). All of these tumours were immunohistochemically positive for HMB-45 and S-100 protein and five of six cases were cytogenetically confirmed by detecting EWS–ATF1 fusion transcripts (table 3).

The rhabdomyosarcomas and poorly differentiated synovial sarcomas were defined morphologically and immunohistochemically, and confirmed by the presence of the PAX3/PAX7–FKHR and SYT–SSX fusion genes by RT-PCR analysis, respectively.

RT-PCR analysis
For the molecular genetic analysis, total RNA was extracted from the frozen tumour tissues using standard procedures.15 The RNA was reverse transcribed, and the samples were then subjected to PCR amplification using a previously described pair of primers for EWS and FLI1,16 EWS–WT1,17 EWS–ATF1,18 PAX3/PAX7 consensus and FKHR,19 and SSX and SYT.20 The PCR was performed according to the methodology described previously in each tumour type.16–20

Fluorescence in situ hybridisation
FISH was carried out according to the manufacturer’s instructions. Briefly, after dewaxing the slides, they were immersed in 0.2N HCl for 20 minutes and pretreatment solution (Vysis, Downer’s Grove, Illinois, USA) at 80°C for 30 minutes, digested with protease for 60 minutes at 37°C, washed in 1× phosphate buffered saline for five minutes at room temperature, fixed in 10% formaldehyde for 60 minutes at room temperature, washed in 1× phosphate buffered saline for five minutes at room temperature, placed into prewarmed denaturation solution (Vysis) for five minutes at 72°C, and then dehydrated by immersing in 70%, 85%, and 100% ethanol for one minute each at room temperature.
and of EWS exon 7 to WT1 exon 8 in the remaining tumour EWS exon 10 to WT1 exon 8 in two tumours (cases 3 and 6), exon 9 to WT1 exon 8 in three tumours (cases 1, 2, and 4), of DSRCTs, the PCR products revealed inframe fusions of EWS transcripts were not detected (cases 2 and 6). In the six one tumour (case 4) (table 1). In two cases, the fusion seven samples. PCR products revealed inframe fusions EWS–FLI1 fusion transcripts were detected in five of these cases of alveolar rhabdomyosarcoma, poorly differentiated synovial sarcomas and three alveolar rhabdomyosarcomas, respectively.

For interphase FISH, the slides were subjected to hybridisation with an LSI EWSR1 (22q12) dual colour, breakapart rearrangement probe (Vysis). The probe consists of a mixture of two FISH DNA probes. The first probe is an ~500 kb probe labelled with spectrum orange; it flanks the 5' side of the EWSR1 gene (22q12) and extends inward into intron 4. The second one is an ~1100 kb probe labelled with spectrum green; it flanks the 3' side of the EWSR1 gene (22q12). The FISH probe mix (10 μl) was added to the sample area of the slides at 45°C. The slides were coverslipped, sealed with rubber cement, and incubated at 37°C for 48 hours in a humidified chamber. The slides were then washed in posthybridisation wash buffer at 72°C. Subsequently, 10 μl of DAPI counterstain was placed on the slide, which was then coverslipped. After hybridisation, all slides were maintained at ~20°C in the dark. Hybridisation signals were visualised with an epifluorescence microscope, and images were captured on a CCD camera. Fifty nuclei that showed both green and orange signals were counted by two different individuals, and the percentages of green, orange, and fused signals were calculated.

RESULTS
Molecular genetic features
Frozen tissues were available from seven of 16 ES/PNETs, and EWS–FLI1 fusion transcripts were detected in five of these seven samples. PCR products revealed inframe fusions between EWS exon 7 and FLI1 exon 5 in four tumours (cases 1, 3, 11, and 14), and EWS exon 7 and FLI1 exon 6 in one tumour (case 4) (table 2). In two cases, the fusion transcripts were not detected (cases 2 and 6). In the six DSRCs, the PCR products revealed inframe fusions of EWS exon 9 to WT1 exon 8 in three tumours (cases 1, 2, and 4), of EWS exon 10 to WT1 exon 8 in two tumours (cases 3 and 6), and of EWS exon 7 to WT1 exon 8 in the remaining tumour (case 5) (table 2). An ampliﬁed PCR product corresponding to the EWS–ATF1 fusion was detected in ﬁve CSSs, three of which were EWS exon 8 to ATF1 exon 4 (cases 1, 5, and 6) and two (cases 2 and 4) were EWS exon 10 to ATF1 exon 5 (table 3). A 583 bp amplification product corresponding to the SYT–SSX fusion gene and an 870 bp amplification product consistent with PAX3–FKHR gene fusion was detected in three poorly differentiated synovial sarcomas and three alveolar rhabdomyosarcomas, respectively.

FISH analysis
Fusion of orange and green signal patterns, as seen in negative control tumour cells, was indicative of an intact copy of the EWS gene. A separated signal pattern of one green and one orange, demonstrating a rearrangement of the EWS gene, was detected in 50–90% of nuclei in 14 of 16 ES/PNETs (table 1) (ﬁg 2A). There was no rearrangement of the EWS gene found in two cases (cases 3 and 9) in which the tissues were decalcified. Fusion signals were detected in more than 90% of the nuclei in all six DRSCTs and in 45–90% of the nuclei in five of the six CSSs specimens (ﬁg 2B, C), but in no cases of alveolar rhabdomyosarcoma, poorly differentiated synovial sarcoma, or neuroblastoma (ﬁg 2D).

DISCUSSION
RT-PCR analysis is a powerful tool for the diagnosis of tumours with chromosomal translocations. However, the limitation of RT-PCR analysis for routine diagnosis is that fresh or frozen tissue is sometimes not available, and that the fusion transcripts are not always detectable with RT-PCR using FFPE tissue. Moreover, it is necessary to prepare various primers, and care needs to be taken to avoid false positive results caused by contamination when carrying out these processes. As seen in our series and that of previous workers,21 22 ES/PNET can rarely occur in old age and in other

<table>
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<th>Table 1: Clinical findings of the 16 ES/PNETs</th>
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<td><strong>Case</strong></td>
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*Decalcified tissue was used.
AWD, alive with disease; DOD, died of disease; FISH, fluorescence in situ hybridisation; NA, data not available; ND, not detected; RT-PCR, reverse transcription polymerase chain reaction.

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<th>Table 2: Clinical findings of the 6 intra-abdominal desmoplastic small round cell tumours</th>
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<td><strong>Case</strong></td>
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AWD, alive with disease; DOD, died of disease; FISH, fluorescence in situ hybridisation; RT-PCR, reverse transcription polymerase chain reaction.
anatomical locations, such as the kidney, duodenum, and pancreas. Diagnosing this tumour in such unusual locations is difficult, and it is necessary to separate it from other solid cancers and malignant lymphoma. Although immunohistochemical analysis of CD99 can provide a clue to the differential diagnosis in this situation, cytogenetic analysis is needed for a definite diagnosis because the expression of CD99 is not specific for ES/PNETs.23

Several investigators have used the FISH technique to identify the 11;22 translocation in ES/PNETs.5–10 However, most of these have been performed on cell lines or touch imprints and short term cultures, and sometimes centromeric and total probes need to be prepared or a computer based statistical analysis is required. These limit the routine clinical use of this technique, as discussed previously.11 In a previous study, a new set of probes specific to both the EWS and FLI1 breakpoint regions was reported to be useful in clinical diagnosis using free nuclear cytospins from FFPE tissue.11 However, this method is relatively labour intensive, and there is the need to eliminate random overlap of signals in non-ES/PNETs.

The newly developed probes that we used were localised to the breakpoints on chromosome 22q12 and provided evidence of the t(22q12) translocation by showing one orange and one green signal pattern on the derivative chromosome 22. The signals generated by these probes were generally large, bright, and easily detectable, and could be detected even when the fusion gene was not detected by RT-PCR (table 1, case 2). These probes provide a definite advantage in the routine clinical diagnosis of screening tumours that share rearrangement of the EWS gene. The average percentage of positive results using these probes for detecting rearrangement of the EWS gene on three tumour types was 90%, indicating an excellent performance. The probes performed extremely well—rearrangements of the EWS gene were detected in approximately 90% of the three tumour types tested. However, this suggests that up to 10% of tumours would not be detected if this test alone was used in clinical diagnosis. Fusion signals were not detected in two ES/PNETs, perhaps because only decalcified tissue was available as the tumours had not extended into the soft tissue in these cases (cases 3 and 6). One case of RT-PCR confirmed CCS was FISH negative; the reasons for the failure of FISH analysis in this case are unclear, but there may have been technical problems because FISH analysis is a multistep procedure.

The gene fusion EWS–FLI1, resulting from t(11;22)(q24;q12), is seen in approximately 85% of cases of ES/PNET. In our series, five of seven (71%) tumours had the EWS–FLI1 translocation. Variant fusions of EWS with other ETS family genes—ERG (at 21q22), ETV1 (7p22), E1AF

<table>
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<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Tumour site</th>
<th>Tumour size (cm)</th>
<th>RT-PCR</th>
<th>FISH</th>
<th>Follow up (months)</th>
<th>Outcome</th>
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<tr>
<td>1</td>
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<td>M</td>
<td>Thigh</td>
<td>4.0</td>
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<td>Positive</td>
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<td>AWD</td>
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<td>Positive</td>
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<tr>
<td>3</td>
<td>38</td>
<td>M</td>
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<td>NA</td>
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<td>252</td>
<td>DOD</td>
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<td>41</td>
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<td>6</td>
<td>AWD</td>
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<tr>
<td>5</td>
<td>62</td>
<td>F</td>
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<td>20</td>
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<tr>
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<td>Hand</td>
<td>NA</td>
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<td>Positive</td>
<td>49</td>
<td>DOD</td>
</tr>
</tbody>
</table>

AWD, alive with disease; CDF, continued disease free; DOD, died of disease; FISH, fluorescence in situ hybridisation; NA, data not available; ND, not detected; RT-PCR, reverse-transcription polymerase chain reaction.
(17q12), and FEV (2q33)—have been found in rare cases of ES/PNET. It is possible that in the two cases (cases 2 and 6) in which we could not detect EWS–FLI1 fusion transcripts, the EWS gene was fused with other ETS variants. Such rearrangements of the EWS gene in ES/PNET were detected in one of the two cases by these probes in FFPE tissue sections.

Although the probes we used specifically identify t(22q12) but cannot specifically identify the translocation partners, there are advantages to detecting t(22q12) translocation by using one probe only: using several probes is costly and time consuming. To distinguish between small round cell tumours sharing EWS rearrangements, it may be necessary to combine both histopathological and immunohistochemical approaches. Moreover, this FISH technique may give either false positive or false negative results, so that it should be carried out together with other cytogenetic analyses, including RT-PCR, in diagnostically challenging cases.

‘The signals generated by these probes were generally large, bright, and easily detectable, and could be detected even when the fusion gene was not detected by reverse transcriptase polymerase chain reaction’

The intra-abdominal DSRCT with multiphenotypic differentiation is a recently identified but well characterised entity with distinctive clinical, light microscopic, and immunohistochemical features.25–30 Cytogenetic studies of DSRCTs have described a consistent chromosomal translocation, t(11;22)(p13;q12), which creates a novel rearrangement of the EWS gene on chromosome 22q12. This method is a useful aid in detecting chromosomal translocation variants in FFPE tissue and will be of value in the clinical diagnosis of ES/PNET and other small round cell tumours sharing EWS rearrangements, including DSRCT and CCS.

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REFERENCES
9 McManus AP, Gusterson BA, Pinkerton CR, et al. Diagnosis of Ewing’s sarcoma and related tumours by detection of chromosome 22q12.

Take home messages
- We devised a fluorescence in situ hybridisation (FISH) technique on formalin fixed, paraffin wax embedded (FFPE) samples of Ewing’s sarcoma/primitive neuroectodermal tumour (ES/PNET) and other small round cell tumours using a commercially available EWSR1(22q12) dual colour, breakapart rearrangement probe
- Interphase FISH using this newly developed probe is sensitive and specific for detecting the EWS gene on FFPE tissues and is useful in the routine clinical diagnosis of ES/PNET and other small round cell tumours, such as desmoplastic small round cell tumour and clear cell sarcoma
- To distinguish between small round cell tumours sharing EWS rearrangements, it may be necessary to combine both histopathological and immunohistochemical approaches

1055
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