Recurrence gains of 1q, 8 and 12 in the Ewing family of tumours by comparative genomic hybridization

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Summary Comparative genomic hybridization (CGH) was used to detect copy number changes of DNA sequences in the Ewing family of tumours (ET). We analysed 20 samples from 17 patients. Fifteen tumours (75%) showed copy number changes. Gains of DNA sequences were much more frequent than losses, the majority of the gains affecting whole chromosomes or whole chromosome arms. Recurrent findings included copy number increases for chromosomes 8 (seven out of 20 samples; 35%), 1q (five samples; 25%) and 12 (five samples; 25%). The minimal common regions of these gains were the whole chromosomes 8 and 12, and 1q21–22. High-level amplifications affected 8q13–24, 1q and 1q21–22, each once. Southern blot analysis of the specimen with high-level amplification at 1q21–22 showed an amplification of FLG and SPRR3, both mapped to this region. All cases with a gain of chromosome 12 simultaneously showed a gain of chromosome 8. Comparison of CGH findings with cytogenetic analysis of the same tumours and previous cytogenetic reports of ET showed, in general, concordant results. In conclusion, our findings confirm that secondary changes, which may have prognostic significance in ET, are trisomy 8, trisomy 12 and a gain of DNA sequences in 1q.

Keywords: Ewing family of tumours; comparative genomic hybridization; 1q; chromosome 8; chromosome 12

Ewing’s sarcoma is the most frequent bone tumour in children under 10 years of age and the third most common primary malignant bone tumour in adults. It is most commonly located in the bone, but it can also arise in soft tissues. Ewing’s sarcoma is closely related to peripheral neuroepithelioma, Askin’s tumour and aesthesioneuroblastoma. These tumours are referred to as the Ewing family of tumours (ET), which characteristically show a high expression of the MIC2 antigen (Ambros et al, 1991).

A specific chromosomal abnormality, t(11;22)(q24;q12), is consistently found in ET (Turc-Carel et al, 1988). It fuses EWS, a previously uncharacterized gene in 22q12, with FLI1 in 11q24 and generates a hybrid transcript (Delattre et al, 1992). In few cases, the EWS gene may be fused with other genes, e.g. ERF on chromosome 21 or ETV1 on chromosome 7 (Zucman et al, 1993; Jeon et al, 1995), both members of the ETS family of transcription factors, like FLI1. The t(11;22) or a variant translocation affecting either 11q24 or 22q12 has been described in 90% of the cases (Mitelman, 1994).

Other chromosomal abnormalities, without the specificity of the primary change, have been detected repeatedly in ET. These secondary changes contribute to tumour progression and may serve as criteria for the aggressiveness of the disease (Mugneret et al, 1988). The most common additional changes are trisomies 8 and 12, and der(1;16). This derivative chromosome often leads to trisomy for 1q. Trisomy 8 has been observed in 44% of the cases (Mugneret et al, 1988), trisomy 12 in 29% (Hattinger et al, 1996) and der(1;16) in 18% (Douglass et al, 1990).

Conventional cytogenetic analysis is often difficult in ET owing to the low number of mitotic cells, poor chromosome morphology and banding, and the complex nature of chromosomal changes. Our aim was to evaluate the incidence of the above-mentioned and other non-random additional changes in ET by comparative genomic hybridization (CGH). CGH makes it possible to identify genomic imbalances with tumour DNA as the only requirement. This method is based on the hybridization of differentially labelled tumour DNA and normal DNA to normal metaphase spreads (Kallioniemi et al, 1992). In the present study, we applied CGH to a series of ETs.

MATERIALS AND METHODS

Tumour specimens

The study was carried out on 20 samples from 17 patients (two specimens from the same patient in three cases). The tumour samples and the respective clinical data are listed in Table 1. Some samples were from frozen tissues and some from paraffin sections (cases 15, 16 and 17). The DNAs from the paraffin-embedded samples were extracted according to the protocol published by Miller et al (1988) with slight modifications. The proportion of tumour cells in the paraffin sections ranged from 70% to 95%. For the fresh samples, it was not possible to obtain the corresponding
Table 1 Clinical characteristics of 20 specimens of the Ewing family of tumours

| Case no. | Age/sex | Primary tumour | Samples | Metastases at diagnosis | Treatment | Survival |
|----------|---------|----------------|---------|-------------------------|-----------|----------|
|          |         |                | P/R/M*  |                         |           |          |
| 1a       | 24/F    | Soft tissue    | P       | Knee region             |           | 46–      |
| 1b       | 24/F    | Soft tissue    | P       | Knee region             |           | 46–      |
| 2a       | 33/M    | Soft tissue    | P       | Calft                   | +(Lung)   | 55–      |
| 2b       | 33/M    | Soft tissue    | P       | Calft                   | +(Lung)   | 55–      |
| 3        | 18/F    | Soft tissue    | P       | Shoulder blade region   |           | 63–      |
| 4        | 27/F    | Bone           | P       | Femur                   |           | 58–      |
| 5        | 18/M    | Bone           | M       | Humerus                 | +(C)      | 71†      |
| 6a       | 36/F    | Soft tissue    | P       | Ankle                   |           | 67–      |
| 6b       | 36/F    | Soft tissue    | P       | Ankle                   | +      | 67–      |
| 7        | 18/F    | Soft tissue    | M       | Spine                   | +(C, R)   | 58†      |
| 8        | 19/F    | Bone           | P       | Pelvis                  |           | 31†      |
| 9        | 49/F    | Bone           | M       | Abdominal subcutis      | +(*)      | 11†      |
| 10       | 33/F    | Soft tissue    | P       | Subcutis, thigh         |           | 86–      |
| 11       | 36/F    | Soft tissue    | P       | Buttock                 |           | 30†      |
| 12       | 12/F    | Bone           | P       | Rib                     | +       | 52–      |
| 13       | 3/M     | Bone           | P       | Ulna                    |           | 38–      |
| 14       | 16/M    | Bone           | R       | Pelvis                  | +(Gastrointestinal) | 33†      |
| 15       | 34/M    | Bone           | R       | Humerus                 | +       | 70†      |
| 16       | 18/M    | Bone           | R       | Femur                   | +       | 32†      |
| 17       | 26/F    | Bone           | P       | Rib**                   |           | 29†      |

*Age at diagnosis in years. F, female; M, male. *Case 6, atypical Ewing's sarcoma; case 9, peripheral primitive neuroectodermal tumour; all the other tumours were typical Ewing's sarcomas. P, primary tumour; R, recurrent tumour; M, metastasis. *Treatment before the operation. C, chemotherapy; R, radiation.

histology, but these samples were always taken with great care from representative areas of the tumours. All cases were re-evaluated by two pathologists (MV and TB) and classified as belonging to the Ewing family of tumours based on histology, staining for the MIC2 gene product (Dako, Glostrup, Denmark) and/or diagnostic findings in the chromosome analysis. Case 6 represents an atypical Ewing's sarcoma, case 9 a peripheral primitive neuroectodermal tumour and the rest typical Ewing's sarcoma (Navarro et al, 1994).

Labelling procedures for CGH experiments

The DNA samples were labelled by direct and indirect methods. Indirect labelling was used for frozen tumour samples and direct for paraffin-embedded tumour samples. In the indirect method, reference DNA from healthy blood donors and tumour DNA were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) and biotin-14-dATP (Gibco BRL, Gaithersburg, MD, USA) respectively. For the direct method, the normal DNA was labelled with Texas red-5-dUTP (DuPont, Boston, MA, USA) and the tumour DNA with fluorescein-12-dUTP (DuPont). Standard nick translation procedures were used in both.

Comparative genomic hybridization

The hybridizations were performed as described by Kallioniemi et al (1994) with some modifications. Briefly, equal amounts of the two DNAs (500 ng) and 10 μg of human Cot-1 DNA (Gibco BRL) were ethanol precipitated and redissolved in 10 μl of 50% formamide/10% dextran sulphate/2× saline sodium citrate (SSC). Normal lymphocyte metaphase preparations were denatured at 68–69°C for 2 min in a formamide solution (70% formamide/2× SSC, pH 7), dehydrated and treated with proteinase K (0.1 μg ml−1 in 20 mM Tris-HCl/2 mM calcium chloride, pH 7) at 37°C for 7.5 min and dehydrated again. The probe mixture was denatured at 75°C for 5 min, applied to the slides and hybridized for 2–3 days at 37°C.

After the hybridization the slides were washed. In indirect labelling, tumour DNA was detected with tetraethylrhodamine isothiocyanate (TRITC) conjugated to avidin, and normal DNA with fluorescein isothiocyanate (FITC) anti-digoxygenin. Chromosomes were counterstained with 10 μg ml−1 4',6-diamidino-2-phenylindole (DAPI) and mounted in an anti-fade solution.

Digital image analysis

The hybridizations were analysed using an Olympus fluorescence microscope and the isis digital image analysis system (MetaSystems, Altussheim, Germany) based on a high-sensitivity integrating monochrome CCD camera and an automated CGH analysis software package.

Interpretation of CGH results and quality control

Ratio profiles were averaged from between five and ten metaphases per sample (up to 20 chromosome homologues). Gains of DNA sequences were defined as chromosomal regions with a fluorescence ratio above 1.17, and losses as regions with a ratio below 0.85. These cut-off values were based on negative control experiments with normal DNAs using both indirect and direct labelling. In these hybridizations, the fluorescence ratios stayed between 0.85 and 1.17. Alternative statistical thresholds based on the r-distribution of the ratio value of balanced chromosomes were also applied. Chromosomal imbalances were confirmed by a 99% confidence
interval. A positive control with known aberrations and a negative control were included in each CGH experiment as quality controls. A ratio over 1.5 was considered to represent a high-level DNA amplification. Heterochromatic regions (1q12, 9q12, 16q11, 13p, 14p, 15p, 21p, 22p and Y chromosome) were excluded from the analysis. The profiles of 1p32–pter, 16p, 17p and chromosomes 19 and 22 were interpreted with caution, because they have been known to give false-positive results (Kallioniemi et al, 1994).

Conventional cytogenetic analysis and interphase hybridization

The methods used for conventional and interphase cytogenetics have been described previously (Tarkkanen et al, 1993).

Southern blot analysis

Preparation of filter blots and hybridization were performed as described previously (Forus et al, 1993). Quantitation of signal intensity was done by two-dimensional densitometry on a Molecular Dynamics laser densitometer. The net signals from specific bands were corrected for unequal sample loading by calibration relative to the signal obtained with an APOB control probe and compared with signals from control samples with a normal copy number of the gene (leucocytes). The probes used from 1q21–22 were a cDNA from the SPRR3 gene (Gibbs et al, 1993; Hohl et al, 1995), kindly provided by Dr Backendorf, and pHc5 FLG (Presland et al, 1992), containing a part of the coding region from the 3′ end of the human filaggrin gene, kindly provided by Drs Fleckman and Presland. A CDNA probe for the APOB gene on human chromosome 2, kindly provided by Dr Breslow (Huang et al, 1985), was used to calibrate for unequal sample loading.

Statistical analyses

The 5-year survival in patients with and without copy number increases in 1q21–22 and in chromosomes 8 and 12 in primary tumours was estimated with the Kaplan–Meier method and the statistical significance tested by the log-rank method. The correlation between overall survival and total number of aberrations in CGH was estimated by the Cox proportional hazards model and the statistical significance with the Wald test.

RESULTS

Comparative genomic hybridization

All DNA sequence copy number changes detected by CGH and chromosome banding data have been listed in Table 2. Fifteen out of the 20 samples (75%) presented DNA sequence copy number changes. Thirteen tumours (65%) showed gains of DNA sequences and five (25%) showed losses. These changes were present at one or more chromosomal sites. On average, there were 2.3 aberrations per sample (range 0–9): 1.9 gains (range 0–9) and 0.4 losses (range 0–2). The mean number of aberrations was 1.5 per sample in primary tumours and in the group of tumour recurrences and metastases. Gains and losses of whole chromosomes or whole chromosome arms were common (71% of all changes). Three tumours showed high-level amplifications (ratio > 1.5). Figure 1 presents the summary of all chromosomal regions with an increased or decreased DNA sequence copy number. The most frequent changes were gains of chromosomes 8 and 12, and gains in the long arm of chromosome 1. Examples of the fluorescence ratio profiles of these chromosomes are illustrated in Figure 2.

### Table 2 CGH and cytogenetic results in 20 samples of the Ewing family of tumours

| Case no. | Cytogenetic data | CGH data |
|----------|------------------|----------|
| 1a       | 50, XX, +8, t(10?):q7,q7, t(11;22)(q24;q12), +12, +14, +21 [21] | +1q21–22, +8, +12, +14q, +21q |
| 1b       | 45–50, XX, +8, t(11;22)(q24;q12), ins [5]/46, XX nca [3] | Normal |
| 2a       | 46, XY, t(11;22)(q24;q12) [1]/46, idem, +der(11,16)(q10;p10), –16 [7] | Normal |
| 2b       | Not available | Normal |
| 3        | 46, XX, del(1)[p;q33?q35], add(11)[q12], add(22)[q12] [10] | Normal |
| 4        | 46–47, XX, –1, –2, –5, add(11)[p15], t(11;22)(q24;q12), +721, +3mar, inc [cp12] | +4q27–33, –q7, –11q21–25, –16 |
| 5        | 46, XY [3] | +14q22–32 |
| 6a       | 46, XX [10] | –3q |
| 6b       | 46, XX [1] | –3q13.3–29 |
| 7        | 49–52, XX, del(2)(p21), +add(5)[p723], del(6)[q12][q16], –9, t(11;22)(q24;q12), der(13;13)(q10;q10), add(14)[q32], –17, add(19)[q13], add(20)[q13], +11, +4–5mar [cp8]/44–46, XX, del(1)[p732?p36], add(4)[p12], del(9)[q22], –14, add(19)[q13], +1–2mar [cp2] | +1q, +5, +8/8q13–24, +12, +20q11.2–13.1 |
| 8        | 46–48, XX, +3–4mar, inc [14] | –1p13–36, +1q21–31, –9p |
| 9        | 47, XX, –4, –10, –15, del(22)[q12], +3–4mar, inc [4] | +1q1q21–22, –6q14–25, +7p22–q11.2, +9q |
| 10       | 42–46, XX, –16, +mar1, +mar2 [cp6]/46, XX [5] | +16q |
| 11       | 47, XX, +mar, inc [cp6]/46, XX [2] | +6, +8 |
| 12       | 46, XX, –1, –11, –22, +mar1, +mar2, +mar3 [cp19] | +1q |
| 13       | 51–54, +8, +c, +D, +mar, inc [11]/46, XY [11] | +2p21–q37, +4, +5p12–15.1, +6, +7, +8, +12, +13q14–15, +18q |
| 14       | 47, XY, +i(1)[q10], t(11;22)(q24;q12) [10] | +6, +12, +21q21–22 |
| 15       | Not available | +4, +8, +12, +14q13–32 |
| 16       | Not available | +4, +8, +12, +14q13–32 |

*CGH, comparative genomic hybridization. †Cases 1–14 from frozen tumour tissue samples and cases 15–17 from paraffin sections. ‡High-level amplifications are shown in bold. *nca, non-clonal aberrations.

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Chromosome 8 was involved in copy number increases in seven tumours (35%). These gains always affected the entire chromosome. One of the tumours with a gain of the whole chromosome 8 had a high-level amplification in 8q13-24. Five tumours (25%) showed a gain in some region of 1q. Three of these were gains of the whole q-arm (two with a high-level amplification), and the other two showed gains at smaller sites. The minimal common region was 1q21-22, highly amplified in two cases. DNA sequences in the long arm of chromosome 1 and the whole chromosome 8 were simultaneously gained in two samples (cases 1a and 7). Five tumours (25%) presented a gain of chromosome 12, always affecting the entire chromosome. The gain of chromosome 12 was accompanied in all cases by a gain of chromosome 8. Copy number increases were also detected in other chromosomal sites, but at a lower frequency. Regions on 4q and 14q showed gains of DNA sequences in three samples each, whereas other chromosomal regions showed copy number increases only in one or two cases.

Regional copy number losses were detected in six different chromosomes, but only in one case each.

**Conventional cytogenetic analysis and interphase in situ hybridization**

Cytogenetic analysis was performed in 16 samples (Table 2). Three tumours (2a, 3 and 4) have been reported previously (Tarkkanen et al, 1993). Clonal aberrations were detected in 13 cases, of which six had the typical t(11;22)(q24;q12). In three tumours, the aberrations involved chromosome 11 and/or 22, indicating most probably the involvement of 11q24 and/or 22q12. In four cases, the exact characterization of the clonal aberrations was not possible owing to poor chromosome morphology and banding and the scarcity of mitotic cells. The der(1;16)(q10;p10) in case 2a was confirmed by interphase in situ hybridization with a centromere-specific probe for chromosome 1 as reported previously (Tarkkanen et al, 1993). Three signals were observed in 30% of the cells.
Southern blot analysis

Case 9 with a high-level amplification at 1q21–22 was analysed for amplification of two genes in 1q21–22, SPRR3 and FLG, which have previously been found to be amplified in some sarcoma samples (Forus et al, 1996). As shown in Figure 3, both genes were amplified: the signal from FLG was 2.7-fold increased compared with the normal sample and the signal from SPRR3 was 2.1-fold increased (i.e. at least five and four copies respectively).

Statistical analyses

The estimated 5-year survival rate was 78% and 50% in cases without and with a copy number increase at 1q21–22 (P = 0.57), 84% and 50% in cases without and with a copy number increase of chromosome 8 (P = 0.16), and 78% and 50% in cases without and with a gain of chromosome 12 (P = 0.3). There was no statistically significant (P = 0.24) correlation between overall survival and the number of aberrations detected by CGH.

DISCUSSION

This study represents the first genome-wide screening of losses and gains of DNA sequences in the Ewing family of tumours (ET). Copy number changes were detected in 15 out of 20 tumours (75%). The most frequent changes include gains of the long arm of chromosome 1 and the whole chromosomes 8 and 12. The low mean number of aberrations, 2.3 per sample, is probably due to the importance of the translocation t(11;22), not detectable by CGH, but may also be caused by normal cell contamination or intratumoral genetic heterogeneity. Copy number changes were detected in all paraffin-embedded tumour samples that are characterized by a high proportion of tumour cells. A gain of chromosome 8 was observed in 35% of the tumours. This abnormality revealed by CGH confirms previous cytogenetic findings: trisomy 8 has been reported in 44% of the ET (Mugneret et al, 1988). Our CGH results suggest that the main region is smaller and located at 8q13–q24, according to the high-level amplification that was found. This area possibly harbours putative oncogene(s) important in the development and progression of ET. Band 8q24 contains MYC, which is known to have an elevated level of expression in Ewing’s sarcoma and related tumours (McKeon et al, 1988). However, it is difficult to establish the role of individual genes when DNA sequence copy number changes affect large regions, because the dosage of numerous genes could be altered simultaneously.

Recurrent gains were also observed in the long arm of chromosome 1 (25%). The gain of DNA sequences in 1q is according to conventional cytogenetic studies of ET. An unbalanced t(1;16) resulting in a non-random derivative chromosome with an extra copy of 1q has been present in 18% of the samples studied (Mugneret et al, 1988; Douglass et al, 1990). By CGH, the minimal common region is 1q21–22. Gains affecting this area have also been reported in different types of soft-tissue sarcoma and in osteosarcoma (Forus et al, 1995a,b; Tarkkanen et al, 1995; Szymanska et al, 1996a). 1q21–22 harbours several genes that may contribute to the development and/or progression of human sarcoma. For example, several members of the S100 family of calcium-binding proteins are clustered on 1q21, e.g. CACY and CAPL, the enhanced expression of which is associated with tumour progression or metastasis (Engelkamp et al, 1993). Recently, the amplification of FLG and SPRR3, located in 1q21, has been reported in some human sarcoma samples (Forus et al, 1996). As shown by the Southern blot analysis, these genes are also amplified in case 9, which by the CGH analysis had a gain in the whole long arm of chromosome 1 with a high-level amplification at 1q21–22.

The present study also revealed gains of the entire chromosome 12 (25%). A recent study reported trisomy 12, detected by conventional cytogenetics and in situ hybridization studies, in 29% of ET (Hattinger et al, 1996). Several oncogenes have been mapped to this chromosome, including SAS, CHOP/GADD153, GLI and A2MR, frequently amplified in human sarcomas (Smith et al, 1992; Forus et al, 1993), and MDM2 and CDK4, known to be amplified also in ET (Ladanyi et al, 1995). Even though all these genes are located in a narrow area (12q13–15) (Mitchell et al, 1995), other studies in soft-tissue sarcomas have also shown other regions of chromosome 12 involved in gains of DNA sequences, such as 12q21–22 and 12q24 (Suijkerbuijk et al, 1994; Forus et al,
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Our CGH analysis shows that many loci frequently show copy number changes in ET. However, the critical and primary event in the tumorogenesis of ET is most likely the t(11;22) or a variant translocation. The secondary abnormalities are the gain of chromosomes 8 and 12, and the gain of DNA sequences in 1q, which agree with cytogenetic studies. As one of the known translocations is a likely primary event, it is possible that these additional changes may have prognostic significance. Owing to the limited number of cases in the present study, testing of the statistical significance of the prognostic effect of these changes is associated with a high risk of a type II statistical error. It is of interest to note, however, that copumber increases in 1q and in chromosomes 8 and 12 were all associated with (non-significant) trends to poor survival. To evaluate this further, a larger number of patients needs to be studied.

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