Virchows Arch (2005) 447: 869–874
DOI 10.1007/s00428-005-0041-0

CASE REPORT

Cecilia Surace · Clelia Tiziana Storlazzi · Jacob Engellau · Henryk A. Domanski · Pelle Gustafson · Ioannis Panagopoulos · Pietro D'Addabbo · Mariano Rocchi · Nils Mandahl · Fredrik Mertens

Molecular cytogenetic characterization of an ins(4;X) occurring as the sole abnormality in an aggressive, poorly differentiated soft tissue sarcoma

Received: 14 March 2005 / Accepted: 29 June 2005 / Published online: 26 August 2005

Abstract Cytogenetic and fluorescence in situ hybridization (FISH) analysis of an aggressive undifferentiated soft tissue sarcoma diagnosed as primitive neuroectodermal tumor (PNET) revealed an insertion ins(4;X)(q31–32;p11p22) as the sole aberration. To identify the molecular genetic consequences, contigs of bacterial artificial chromosomes (BACs) covering Xp11–p22 and 4q31–32 were constructed. The breakpoint in Xp22 was considered unlikely to be of pathogenetic significance, as it was very close to the Xp telomere, a region devoid of known or predicted genes. The breakpoint in Xp11 was mapped within a BAC clone containing *BCOR*, encoding a BCL6 (B-cell lymphoma 6)-interacting protein that may influence apoptosis, as the only known gene. FISH analysis with three overlapping clones on normal chromosomes 4 disclosed that the insertion of Xp11 material in der(4) was accompanied by a deletion of chromosome 4 material. Only a predicted gene (XM_094074) was shown to be partially included in the deletion. This gene displays a high similarity with the gene encoding the embryonic blastocoelar extracellular matrix (ECM) protein in sea urchin, which is involved in the migration of the primary mesenchyme cells during embryogenesis. Our results suggest that BCOR and/or an ECM-like protein could be involved in the pathogenesis of a subgroup of PNET or PNET-like sarcomas.

Keywords Cytogenetics · In situ hybridization · Sarcomas · Cancer

C. Surace · I. Panagopoulos · N. Mandahl · F. Mertens (✉)
Department of Clinical Genetics,
Lund University Hospital,
SE-221 85 Lund, Sweden
e-mail: fredrik.mertens@klingen.lu.se
Tel.: +46-46-173387
Fax: +46-46-131061

C. Surace · C. T. Storlazzi · P. D'Addabbo · M. Rocchi
Department of Genetics and Microbiology
University of Bari,
Bari, Italy

J. Engellau
Department of Oncology,
University Hospital,
Lund, Sweden

H. A. Domanski
Department of Pathology,
University Hospital,
Lund, Sweden

P. Gustafson
Department of Orthopedics,
University Hospital,
Lund, Sweden

Introduction

Cytogenetic analyses of soft tissue tumors have disclosed several chromosomal translocations, often seen as the sole karyotypic aberration, that are strongly associated with the morphology of the tumor cells [10]. At the molecular level, these translocations often result in the creation of fusion genes, which seem to be equally strongly associated with histologic tumor type. Cytogenetic or molecular genetic information may thus provide diagnostically valuable information. This is especially true for poorly differentiated sarcomas occurring in children and adolescents, where it sometimes may be difficult to differentiate between, for example, synovial sarcoma, rhabdomyosarcoma, and primitive neuroectodermal tumor (PNET) on the basis of morphologic, ultrastructural, and immunohistochemical features alone. It is, however, vital to reach a correct diagnosis, as the different tumor types carry different prognoses and require different treatment strategies.

In the present study, we report the detailed molecular cytogenetic characterization of a previously unreported recombination between chromosomes X and 4, seen as the only cytogenetic aberration in short-term cultured cells from an aggressive undifferentiated soft tissue sarcoma occurring in an 18-year-old boy.

Clinical history

A previously healthy 18-year-old boy had for 3 months noticed increasing pain from a growing mass in his right elbow. A radiograph showed a 6-cm periosteal reaction of the proximal ulnar bone with possible cortical resorption, and MRI displayed a 10×7×3-cm tumor in the proximal ulnar diaphysis and infiltration of adjacent soft tissues. There were no signs of engagement of the bone marrow or of metastasis to the lymph nodes in the axilla. A pulmonary CT showed multiple nodules up to 0.5 cm in diameter in both lungs, but there were no signs of metastases to the mediastinal lymph nodes. A total-body radionuclear bone scan revealed increased activity only in the right elbow. A fine-needle aspiration biopsy (FNA) showed round-to-oval, often dissociated cells, a high mitotic index, and focal areas of necrosis. The tumor cells stained positive for CD99 and vimentin. Electron microscopy showed that the tumor cells displayed sparse, often extended cytoplasm, abundant mitochondria, and small organelles containing glycogen and fat. Based on these findings, the patient was diagnosed with a primitive neuroectodermal tumor (PNET). Neoadjuvant chemotherapy was initiated with vincristin (1.5 mg/m^2), doxorubicin (30 mg/m^2), ifosfamide (1,000 mg/m^2), and cisplatinum (90 mg/m^2) according to the Scandinavian Sarcoma Group protocol IX for soft tissue sarcomas. The chemotherapy was administered without significant subjective toxicity. After four cycles of treatment, a pulmonary CT showed only a minimal mass remaining in the right apical lobe, and MRI of the extremity revealed a marked regression of the primary tumor. The patient underwent surgery with en bloc resection with surgically wide margins of the primary tumor. Both lungs were explored, and the solitary remaining mass in the upper right lobe was excised. Histological examination of the primary tumor showed large areas of fibrosis and small foci of necrosis, probably due to the preoperative chemotherapy. A few small, scattered areas of viable tumor cells were found. Immunostaining showed focal positivity for CD99, while keratins and EMA were both negative. The response to the preoperative chemotherapy was classified as poor (Huvos grade III). No viable tumor cells were found in the resected lung metastasis. Treatment was then continued with one more cycle of chemotherapy, after which stem cell mobilization was intended, followed by myeloablative treatment with high-dose busulphan-melphalan. However, the patient refused further chemotherapy. Instead, the patient received total lung irradiation, with 15 Gy to both lungs (1.25 Gy per daily fraction with 6 MV photon energy).

On a pulmonary CT in October 1999, 17 months after diagnosis, metastases to the lungs were suspected, but the patient refused further examination until January 2000, when metastasectomy of all evident pulmonary metastases was performed. In sections from the lung metastases, the morphology of the tumor tissue was better preserved than in the primary tumor, and diffusely infiltrating tumor cells, having sparse-to-moderate cytoplasm and rounded-to-oval nuclei with finely granular, clumped chromatin, somewhat irregular nuclear membrane, and inconspicuous nucleoli, were seen. Numerous mitoses were seen, as well as small foci of apoptotic cells (Fig. 1). In addition, there were areas where cellular pleomorphism was more marked, with larger cells and more prominent anisokaryosis, irregular cell membranes and coarser nuclear chromatin (Fig. 2). Rosettes, perivascular pseudorosettes, or cytoplasmic glycogen were not seen. Tumor cells were negative for CD99, keratins, and EMA.

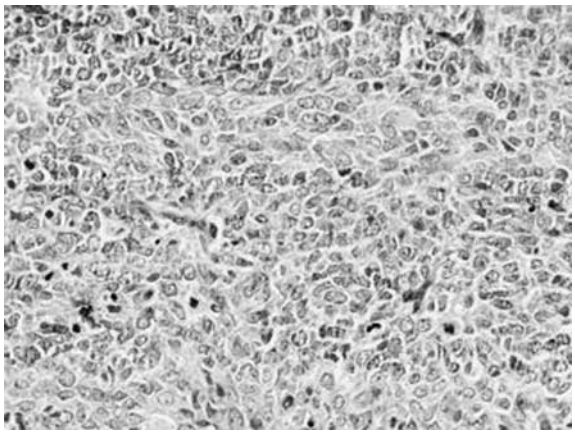

Fig. 1 Histologic section from lung metastasis showing diffusely infiltrating, relatively uniform, or moderately pleomorphic tumor cells with an admixture of apoptotic cells (H & E, ×50)

At this time, the patient refused chemotherapy. New lung metastases were detected 3 months later, and after an additional 2 months, four cycles of high-dose ifosfamide (2,000 mg/m^2) were given. No signs of tumor response were seen, and the patient was subsequently treated from September 2000–May 2001 with oral trofosfamide-etoposide. Radiotherapy to the mediastinum (30 Gy in ten fractions) and apical thoracic cavity (20 Gy in five fractions) was given, but in November 2001, the patient died of respiratory insufficiency due to massive intrathoracic tumor growth.

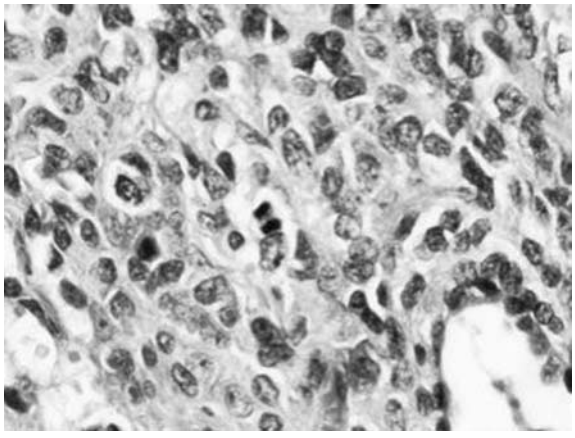

Fig. 2 High-power view from areas with more pleomorphic tumor cells showing prominent anisokaryosis and coarse nuclear chromatin (H & E, ×100)

Materials and methods

Cell culturing, harvesting, and G-banding were performed as previously reported [8]. The karyotype was described according to the International System for Human Cytogenetic Nomenclature [7].

The following bacterial artificial chromosome (BAC) clones were used as probes for fluorescence in situ hybridization (FISH) analysis in order to reveal the exact location of the breakpoints on the X chromosome (the first ten clones) and chromosome 4 (the remaining 15 clones): RP11-552J9 (accession no. AL450023); RP11-344N17 (AL606490); RP11-552E4 (AL683817); RP11-38O23 (AL356464); RP11-386N14 (AL133545); RP11-291H16 (clone placed on cytogenetic map using the STS marker RH63518); RP11-429N5 (genomic survey sequence gi|11995676|gb| AZ254511.2); RP11-495K15 (genomic survey sequence gi|11500710|gb|AZ303207.2); RP11-87M18 (AL606516); RP11-215A12 (AC084716); RP11-196J14 (AC024032); RP11-83A24 (AC097376); RP11-364L4 (AC097658); RP11-58H15 (AC104685); RP11-481K16 (AC139713); RP11-10M9 (AC018535); RP11-424A19 (genomic survey sequence gi|21955327|gb|BH861015.1); RP11-780M14 (AC104090); RP11-673E1 (AC093890); RP11-291L15 (AC098588); RP11-543H9 (AC096757); RP11-552I10 (AC093863); RP11-667D12 (AC093887); RP11-461L13 (AC080078); RP11-458C23 (genomic survey sequence gi|5009527|gb|AQ582417.1). For each chromosome, the BACs are listed according to their map position, starting with the most proximal one. Their location was defined according to the UCSC database (University of California Santa Cruz, http://genome.ucsc.edu/index.html, May 2004 release). The clones belong to the RPCI library (http://www.chori.org/bacpac/) and were obtained from Resources for Molecular Cytogenetics (http://www.biologia.uniba.it/rmc/). Whole chromosome painting (WCP) probes for the X chromosome and chromosome 4 were obtained from Vysis (Downers Grove, IL, USA) and Cambio (Cambridge, UK). Probes were directly labeled with Cy3-dUTP (Amersham Biosciences, Little Chalfont, UK) and Fluor-X-dCTP (Amersham Biosciences) and indirectly labeled with biotin-dUTP (Roche, Penzberg, Germany) and detected with streptavidin-diethylaminocoumarin (Roche and Molecular Probes, Leiden, The Netherlands). The labeling of all the clones was performed by use of Amersham's Mega Prime kit (Amersham Biosciences). FISH was carried out as described elsewhere [5]. The hybridizations were analyzed with the aid of the Chromofluor System (Applied Imaging, Newcastle, UK). The BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) was used for computer analysis of nucleotide and protein sequence data, in order to look for candidate genes potentially involved in the breakpoints of the chromosome rearrangement.

In order to exclude cryptic rearrangement of the *EWSR1* gene, metaphase-FISH was performed with BACs flanking

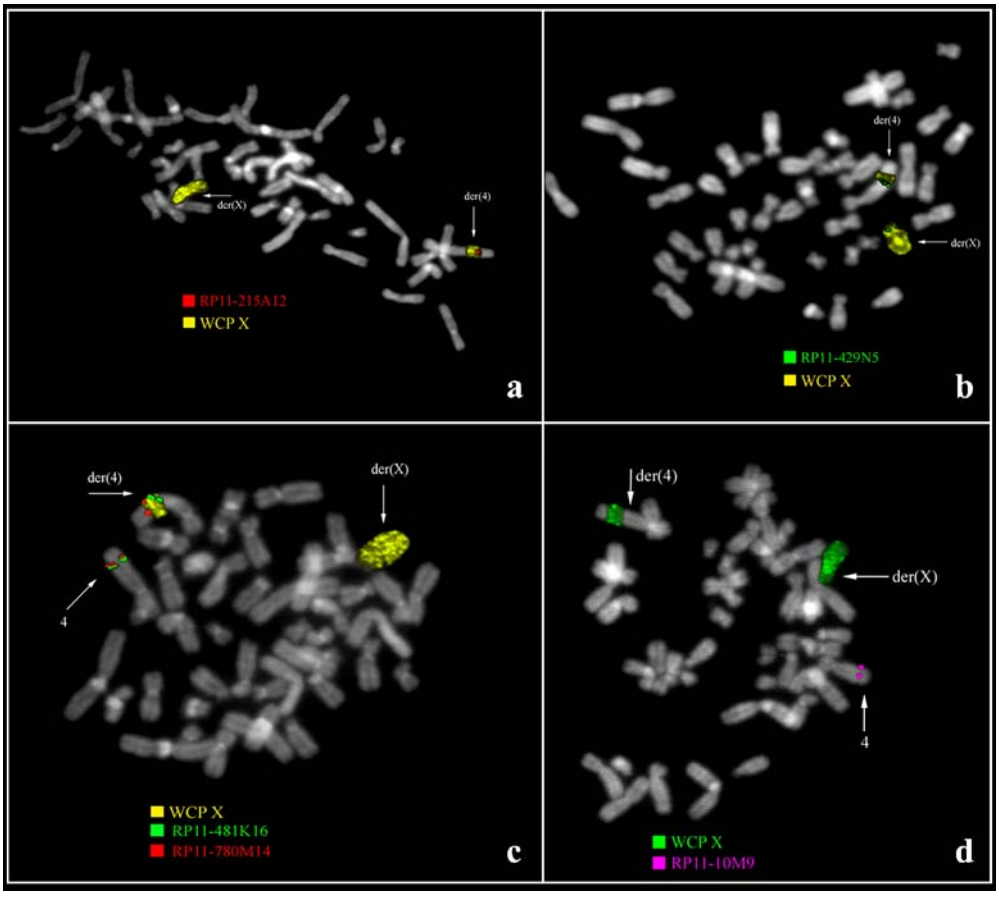

Fig. 3 Results of FISH experiments. The WCP probe for the X chromosome was cohybridized with the following probes: subtelomeric BAC specific for Xp (RP11-215A12) (**a**); BAC RP11-429N5, containing the breakpoint on the X chromosome (**b**); and RP11-481K16 (**c**), RP11-780M14 (**c**), and RP11-10M9 (**d**), covering the region containing the putative breakpoint on the chromosome 4

the *EWSR1* locus (data not shown). The presence of a cryptic *SS18/SSX* fusion gene was excluded through RT-PCR analysis as described [12].

Results

G-banding analysis of metaphase spreads from short-term cultured cells from a fine-needle aspiration biopsy obtained before treatment, as well as from the excised primary tumor obtained after chemotherapy, revealed the following karyotype: 46,Y,t(X;4)(p11;q31)/46,XY. Cytogenetic analysis of peripheral blood lymphocytes showed a normal male chromosome complement. RT-PCR did not reveal any *SS18/SSX* fusion transcript, and FISH for *EWSR1*-rearrangement was negative (data not shown). Preliminary FISH experiments using the WCP probes for X and 4 showed that the chromosomal rearrangement was an inser-

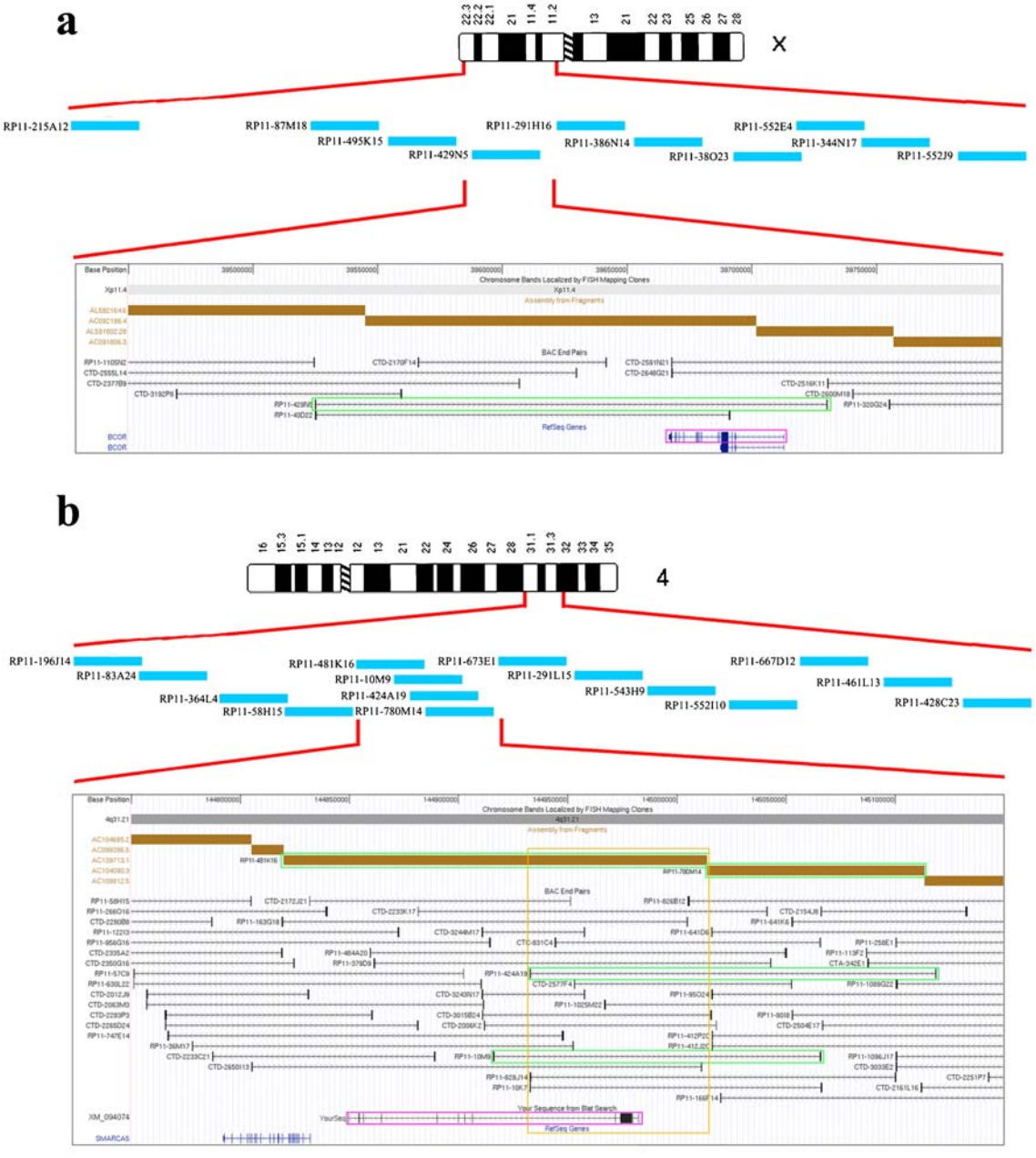

Fig. 4 Location of the clones used in FISH experiments. BACs included in the studied regions (*red intervals* starting from chromosomes) were represented as *light blue boxes*. Regions containing the breakpoints (*red intervals* starting from BACs) were enlarged, and UCSC database web pages were reported (University of California Santa Cruz, http://genome.ucsc.edu/index.html, May 2004 release). The clone RP11-429N5, containing the breakpoint on the X chromosome, was surrounded by *green frame*, and the sole gene included in this area (*BCOR*) was enclosed in a *purple rectangle* (**a**). The BACs covering the breakpoint region on the chromosome 4, RP11-481K16, RP11-10M9, RP11-424A19, RP11-780M14, were displayed surrounded by *green borders*, and the predicted gene (XM_094074) included in this region was enclosed in a *purple frame*. The deleted area was bounded by a *yellow rectangle* (**b**)

tion of Xp into 4q, rather than a translocation. Further FISH analysis revealed that the part of the X chromosome inserted into chromosome 4 spanned from Xp11 to Xpter because the signal for the Xp subtelomeric probe RP11-215A12 was seen on the der(4) (Fig. 3a). Based on these findings, the revised abnormal karyotype was 46,Y, ins(4;X)(q31–32;p11p22).

Contigs of BACs spanning chromosome bands Xp11–Xp21.1 and 4q31–4q32.1 were constructed as described in Materials and methods. The salient findings were as follows: FISH with the X-chromosome-specific clone RP11-429N5 gave a split signal on the derivative chromosomes X and 4 (Fig. 3b). Hence, the breakpoint on X could be mapped to sub-band Xp11.4, between the base positions identified by the ends of the BAC clone. None of the BACs selected for chromosome 4 resulted in a split signal, but the clones RP11-481K16 and RP11-780M14, which are adjacent on normal chromosomes 4, were separated on the der(4) because of the insertion of Xp-material (Fig. 3c). Moreover, FISH with the clone RP11-424A19, which partially overlaps both these clones, resulted in a single signal on the der(4), on the same, telomeric side of the insertion as RP11-780M14. Based on these results, the breakpoint could be mapped to the region between the centromeric end of RP11-424A19 and the telomeric end of RP11-481K16. The fact that we did not observe any splitting of signals using these clones suggested that a segment of that region was deleted. Indeed, FISH analysis with the clone RP11-10M9 did not give any detectable signal on the der(4) (Fig. 3d).

Discussion

In the present study, we report the molecular cytogenetic characterization of an aggressive undifferentiated soft tissue sarcoma diagnosed as PNET. The sole cytogenetic change detected at G-banding analysis, interpreted as a t(X;4), could after FISH analysis be shown to represent an insertion of Xp-material into the long arm of chromosome 4: ins(4;X)(q31–32;p11p22). Neither an ins(4;X) nor a t(X;4) with these or similar breakpoints has previously been reported in soft tissue tumors [10]. However, since this insertion was the sole cytogenetic abnormality, it is reasonable to assume that it was pathogenetically important, and we thus decided to investigate its molecular genetic consequences in more detail.

The X chromosome had two breakpoints, both of which potentially could have harbored genes of importance for tumor development. However, the breakpoint in Xp22 could be shown to be distal to the subtelomeric region recognized by BAC RP11-215A12, making it highly unlikely that the Xp22 breakpoint was of pathogenetic significance. The breakpoint in Xp11 was defined by BAC clone RP11-429N5, showing split signals on the derivative chromosomes X and 4. In the UCSC (May 2004 release) and Ensembl (http://www.ensembl.org/; April 2004 release) databases, there is only one known gene, *BCOR*, mapped to the region covered by this clone (Fig. 4a). *BCOR* encodes a BCL6 (B-cell lymphoma 6)-interacting corepressor that may influence apoptosis. Aberrant expression of *BCL6* due to chromosomal translocations is seen in a subset of non-Hodgkin's lymphoma [6], and BCOR binds AF9, which is a common *MLL* fusion gene partner in acute myeloid leukemia, suppressing AF9 transcriptional activity [13]. *BCOR* mutations have also been implicated in two human syndromes (oculofaciocardiodental syndrome and Lenz microphthalmia), and knock-out experiments performed on the zebrafish ortholog of *BCOR* showed developmental perturbations consistent with the human syndromes, confirming that BCOR is a key transcriptional regulator during early embryogenesis [11]. Based on this previous information, and the fact that there are no other known or predicted genes in the region covered by RP11-429N5, *BCOR* comes forth as a good candidate target gene on the X chromosome.

Regarding the characterization of the breakpoint on chromosome 4, the results suggested that the insertion was accompanied by a deletion of chromosome 4 material. We investigated this region in order to check for putative target genes. No known gene was found, but a gene predicted by Genescan (accession XM_094074.7) was partially included in the deleted region (Fig. 4b). The protein encoded by this predicted gene belongs to a family of proteins conserved from invertebrates to man, whose only member already studied is the embryonic blastocoelar extracellular matrix protein 3 (ECM3, 47% identity with the human protein) of sea urchin (*Lytechinus variegatus*), a protein that is involved in the migration of the primary mesenchyme cells during sea urchin embryogenesis [4]. Furthermore, the ECM3 protein has a large N-terminal NG2-like domain that is related to NG2, a mammalian chondroitin sulfate proteoglycan core protein, which can induce the spreading and migration of other cell types and has been found overexpressed in many human melanomas [1–3]. In addition, the BLASTP analysis of the predicted protein disclosed a 29% identity with the protein encoded by the human *FRAS1* gene that belongs to a family of proteins related to the ECM protein in sea urchin [9].

Unfortunately, lack of material precluded any attempt to investigate the involvement of any of the candidate target genes at the molecular level. However, our results strongly suggest that a subset of undifferentiated, PNET-like soft tissue sarcomas develop through aberrant expression of the *BCOR* gene on chromosome X and/or a gene encoding an ECM-like protein on chromosome 4, and rearrangements of these genes should be searched for in PNET or undifferentiated sarcomas that do not express any of the known fusion genes associated with PNET, synovial sarcoma, or alveolar rhabdomyosarcoma.

Acknowledgements This work was supported by the Swedish Cancer Society, the Swedish Children's Cancer Foundation, Associazione Italiana per la Ricerca sul Cancro (AIRC), and Centro di Eccellenza Geni in campo Biosanitario e Agroalimentare (CEGBA). Cecilia Surace was supported by a Fondazione Italiana per la Ricerca sul Cancro (FIRC) fellowship. The experiments performed comply with the Swedish and Italian laws.

References

1. Burg MA, Nishiyama A, Stallcup WB (1997) A central segment of the NG2 proteoglycan is critical for the ability of glioma cells to bind and migrate toward type VI collagen. Exp Cell Res 235:254–264
2. Burg MA, Grako KA, Stallcup WB (1998) Expression of the NG2 proteoglycan enhances the growth and metastatic properties of melanoma cells. J Cell Physiol 177:299–312
3. Burg MA, Pasqualini R, Arap W, Ruoslahti E, Stallcup WB (1999) NG2 proteoglycan-binding peptides target tumor neovasculature. Cancer Res 59:2869–2874
4. Hodor PG, Illies MR, Broadley S, Ettensohn CA (2000) Cell-substrate interactions during sea urchin gastrulation: migrating primary mesenchyme cells interact with and align extracellular matrix fibers that contain ECM3, a molecule with NG2-like and multiple calcium-binding domains. Dev Biol 222:181–194
5. Höglund M, Johansson B, Pedersen-Bjergaard J, Marynen P, Mitelman F (1996) Molecular characterization of 12p abnormalities in hematologic malignancies: deletion of KIP1, rearrangement of TEL, and amplification of CCND2. Blood 87:324–330
6. Huynh KD, Fischle W, Verdin E, Bardwell VJ (2000) BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev 14:1810–18233
7. ISCN (1995) An international system for human cytogenetic nomenclature. Mitelman F (ed). Karger, Basel
8. Mandahl N, Heim S, Arheden K, Rydholm A, Willen H, Mitelman F (1988) Three major cytogenetic subgroups can be identified among chromosomally abnormal solitary lipomas. Hum Genet 79:203–208
9. McGregor L, Makela V, Darling SM, Vrontou S, Chalepakis G, Roberts C, Smart N, Rutland P, Prescott N, Hopkins J, Bentley E, Shaw A, Roberts E, Mueller R, Jadeja S, Philip N, Nelson J, Francannet C, Perez-Aytes A, Megarbane A, Kerr B, Wainwright B, Woolf AS, Winter RM, Scambler PJ (2003) Fraser syndrome and mouse blebbed phenotype caused by mutations in FRAS1/Fras1 encoding a putative extracellular matrix protein. Nat Genet 34:203–208
10. Mitelman F, Johansson B, Mertens F (eds) (2001) Mitelman database of chromosome aberrations in cancer. http://cgap.nci.nih.gov/Chromosomes/Mitelman
11. Ng D, Thakker N, Corcoran CM, Donnai D, Perveen R, Schneider A, Hadley DW, Tifft C, Zhang L, Wilkie AO, van der Smagt JJ, Gorlin RJ, Burgess SM, Bardwell VJ, Black GC, Biesecker LG (2004) Oculofaciocardiodental and Lenz microphthalmia syndromes result from distinct classes of mutations in BCOR. Nat Genet 36:411–416
12. Panagopoulos I, Mertens F, Isaksson M, Limon J, Gustafson P, Skytting B, Akerman M, Sciot R, Dal Cin P, Samson I, Iliszko M, Ryoe J, Debiec-Rychter M, Szadowska A, Brosjo O, Larsson O, Rydholm A, Mandahl N (2001) Clinical impact of molecular and cytogenetic findings in synovial sarcoma. Genes Chromosomes Cancer 31:362–372
13. Srinivasan RS, de Erkenez AC, Hemenway CS (2003) The mixed lineage leukemia fusion partner AF9 binds specific isoforms of the BCL-6 corepressor. Oncogene 22:3395–3406