

New Insights into the Cellular Pathways Affected in Primary Uterine Leiomyosarcoma

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Abstract. Resistance to chemotherapeutic agents and radiotherapy has kept surgery the primary treatment of uterine leiomyosarcoma (ULMS). In search of leads for potential therapeutic targets, array CGH (aCGH) was used to obtain a genomewide pattern of ULMS-specific genetic imbalances and to define the affected biological processes. Fine-resolution genomewide aCGH analysis was performed using customised 16K cDNA microarrays on 18 primary ULMS cases. Furthermore, patterns of DNA copy number changes were assessed for associations with clinical parameters, i.e., tumour grade, tumour size and patient status at last follow-up. Our aCGH results demonstrated extensive DNA copy number changes in all chromosomes. Of the 10,590 gene loci included in the analysis, 4,387 were found to be affected by DNA copy number gains and 4,518 by DNA copy number losses in at least one case. Further analyses revealed that 231 of these were commonly gained, and 265 lost in at least 20% of the cases. The gains affected loci at 1p, 1q, 2p, 3p, 6p, 8q, 10q and 18q, whereas losses were observed at 2q, 4q, 6p, 6q, 7p, 7q, 13q, 14p, 16q, 19p, Xp and Xq. Enrichment analysis of biological

processes revealed the gained genes to be involved in the G1/S transition of mitotic cell cycle, co-translational protein targeting to membrane, actin filament polymerisation and positive regulation of cytokine biosynthesis, whereas the genes affected by losses were associated with DNA replication, chromatin modification, telomere maintenance, meiosis, mitosis and angiogenesis. These biological processes featured prominently two well-established tumour suppressors (BRCA2, EREG) and one proto-oncogene (GFII). No statistically significant associations were found between the aberration patterns and clinical variables. Analysis of gene pathways using aCGH uncovered the biological networks involved in malignant progression of ULMS.

Leiomyosarcomas (LMS) are uncommon malignant smooth-muscle tumours that occur in the uterus and soft tissues. LMS is the third most common type of sarcoma of soft tissues after malignant fibrous histiocytoma and liposarcoma (1). Histologically the tumours are composed of spindle cells with blunt-ended nuclei and eosinophilic cytoplasm. Immunohistochemically LMS is characterized by expression of alpha-smooth muscle-actin (SMA) (2), desmin (3) and h-caldesmon (4).

Uterine leiomyosarcoma (ULMS) is a rare gynaecologic malignancy with poor prognosis. ULMS account for approximately 40% of uterine sarcomas and 1% of all uterine cancers. The rate of recurrence is high, ranging from 45% to 73%. Overall five-year survival rates have been reported at 25% to 75% (5). As this sarcoma is rather resistant to chemotherapeutic agents and radiotherapy, surgery remains the primary treatment regimen. The origin of ULMS may be smooth-muscle cells of uterus or

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malignant transformation of a pre-existing leiomyoma (6).

So far, conventional comparative genomic hybridization (cCGH) has been used to identify gains, losses and high-level amplifications of small chromosomal areas in 32 primary ULMS (7-9). Extensive genetic imbalances were detected in nearly all cases. The most common alterations were gains of 1q, 17p and Xp, and losses of 2p, 10q, 12p, 13q, 14q, 16q, 21 and 22q. Our laboratory has recently reported a cCGH analysis of a series of 51 primary LMS, including 19 ULMS and 32 other LMS cases (10). Genomic imbalances were found in 94% of the cases. The most frequent DNA copy number alterations were losses in 10q, 13q and 16q, gains in 1q and high-level amplifications in 17p. A recent analysis of seven ULMS cases by array CGH (aCGH) with 1,440 BAC clones revealed that the percentage of average losses (15.1%) was significantly higher than that of gains (4.86%) (11).

In the present study, we performed a high-resolution analysis of DNA copy number changes in 18 primary ULMS using cDNA-based array aCGH. We used microarrays with 16,000 cDNA clones spotted in duplicates, targeting genes throughout the genome to obtain a genome-wide pattern of chromosomal imbalances and to define the biological processes affected by DNA copy number changes. Furthermore, patterns of DNA copy number changes were assessed for associations with clinical parameters, *i.e.*, tumour grade, tumour size and patient status at last follow-up.

Materials and Methods

Tumour specimens. The tumour material used in this study was previously analysed by cCGH (10). The material of the current study consisted of 18 primary uterine LMS samples obtained from 18 Finnish patients treated at the Helsinki University Central Hospital, Helsinki, Finland. An experienced sarcoma pathologist (R.B.) performed the histopathological review to confirm the diagnosis and to re-evaluate the tumour grading. A two-grade system was applied to classify the tumours as low-grade or high-grade. The main criteria for malignancy grading were the degree of cellular atypia, mitotic activity and proportion of tumour necrosis. Of the 18 samples, eight (44%) were low-grade and ten (56%) were high-grade. The median age of the patients at diagnosis was 57 years (range, 34-81 years). The size of the tumours varied from 4.0 cm to >20.0 cm, with a median tumour size of 7.0 cm. The median follow-up of the patients was 37.2 months, ranging from 3.6 months to 121.7 months. Six patients (33.0%) developed a metastatic course of disease during the follow-up. None of the patients had received chemo- and/or radiotherapy before surgery. Complete clinical and follow-up data have been reported by Svarvar *et al.* (10).

Array CGH (aCGH). Array CGH was performed on customised cDNA 16K microarrays containing 16,000 annotated gene probes in duplicates (National Microarray Resource Center, Turku, Finland; <http://microarrays.btk.fi>). The analysis was performed, as described previously (12-14). DNAs were digested using Alu I

and Ras 1 restriction enzymes (80 U/ μ l). The digested tumour and reference DNAs (6 μ g each) were labelled by random priming, as described previously (14, 15). The slides were hybridised for 17 h at 65°C in a water bath and post-hybridisation washes were performed in 0.5xSSC/0.1% SDS (15 min), 0.5xSSC/0.1%SDS (15 min), 0.5xSSC/0.01% SDS (15 min), 0.06xSSC (2 min) and finally in 0.06xSSC (2 min). Slides were dried and scanned using an Agilent laser confocal scanner and the images were analysed using Agilent G2565AA Feature Extraction Software (Agilent Technologies, Palo Alto, CA, USA). Genes were mapped to the clones presented on the arrays using software developed by the Biomedicum Bioinformatics Unit (University of Helsinki, Helsinki, Finland). In brief, the nucleotide sequences for all the clones spotted onto the array were retrieved from the GenBank database and blasted against the most recent version of the human genome assembly. The blasting algorithm employed was optimised for window size and handling of multiple hits, allowing the flagging of clones whose physical location was ambiguous so that they could be excluded from further analysis.

Statistical analysis

Identification of gains and losses. Output metrics from the Agilent Feature Extraction software were imported into the GeneSpring 7.2 software (Redwood City, CA, USA) and low quality data was removed through a rigorous filtering process. For each array the "R surrogate used", "G surrogate used", "gls found" and "rls found" flags, as well as all 8 "outlier" flags, were required to be 0. Genes with missing values in $\geq 50\%$ of the samples were removed. Intensity ratios were transformed using base 2 logarithm and missing values were imputed by the array mean. Identification of statistically significantly gained or lost chromosomal loci was performed using CGH-Explorer 2.5 at a false discovery rate (FDR) of 1%.

Associations to clinical data. A data set was constructed where the gain/loss status of each gene was coded as one (1) for gain, minus one (-1) for loss, and zero (0) for absence of any DNA copy number changes. Aberration patterns with association to tumour grade, tumour size dichotomized into small (≤ 10 cm) and large (> 10 cm), and status at last follow-up (death *vs.* no evidence of disease) were evaluated using multivariate statistics. A matrix representing all possible pair-wise similarities was first calculated using Pearson's correlation. The most significant underlying dimensions of the highly complex matrix data set were then extracted by multidimensional scaling, allowing samples to be projected onto a reduced space composed of the three dimensions yielding the maximum goodness of fit with the original data set. Clusters associated with any given clinical factor analysed were identified by labelling the data points that represented samples at the same level of a given clinical factor.

Analysis of biological associations. Lists of genes with statistically significant gains or losses in at least 20% of samples were assessed for biologically relevant associations using the WEBGESTALT tool (<http://genereg.ornl.gov/webgestalt/>). Annotation information defining the biological processes to which each gene could be ascribed was retrieved from the classifications provided by the GeneOntology (GO) Consortium (16). Hypergeometric test was performed to assess the degree of

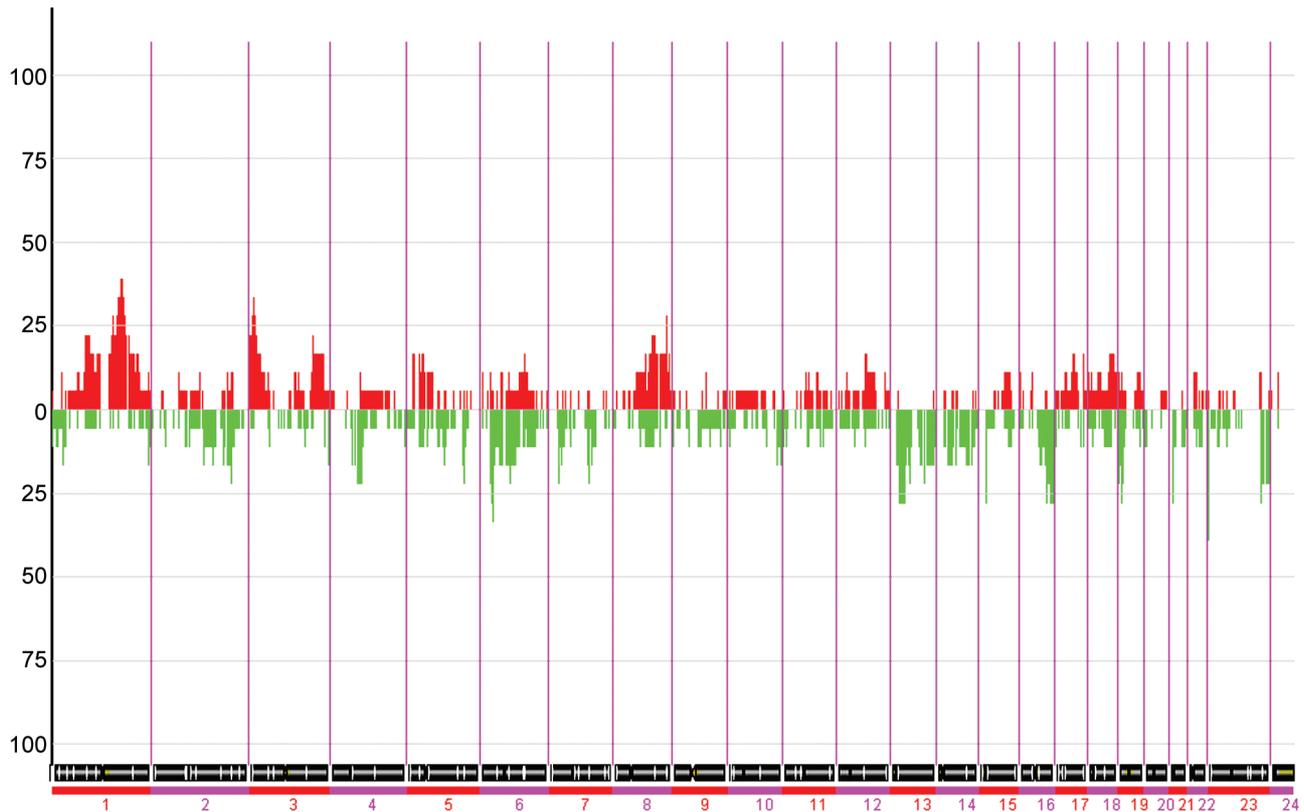


Figure 1. Summary plot showing the proportion of primary uterine leiomyosarcoma samples with statistically significant ($FDR < 0.01$) gains and losses for all chromosomes, aligned from 1 pter to Y qter.

enrichment of genes in various processes. Combined with the requirement that the category or pathway includes at least two of the listed genes, a p -value < 0.01 was considered indicative of statistically significant enrichment. To visualise the structure of relationships between enriched GO categories, the results were depicted in directed acyclic graphs.

Comparison between cCGH and aCGH results. The aCGH results obtained in the present study were compared to previous cCGH results by Svarvar *et al.* (10). To make the comparisons feasible, the aCGH results had to be adjusted to match the lower resolution scale of cCGH. To this end the scores obtained for all gene loci within each of the cytogenetic bands ($n=387$) were averaged. Positive and negative averages were considered indicative of a predominance of gains or losses, respectively, in that chromosome segment, while an average equalling zero signified that no aberrations were found in the region. The ambiguity in representing chromosome bands that exhibited an equal number of loci with losses and gains was avoided by simply excluding those bands from further analysis. Pairwise correlations between all 36 samples, 18 assessed using cCGH and 18 using aCGH, were calculated according to Pearson's method. To visualise the results a hierarchical tree was constructed employing unweighted pair-group average linkage amalgamation rules.

Results

DNA copy number changes revealed by aCGH. Complex patterns of DNA copy number changes were observed throughout the genome in all 18 ULMS (Figure 1; Appendix 1). Of the 10,590 genes included in the analysis, 4,387 were gained and 4,518 lost in at least one case. To focus on the most representative copy number alterations, a threshold was set to include in further analysis only the alterations that were shared by at least 20% of the cases. This approach identified a total of 231 commonly gained genes (Appendix 3) and 265 commonly lost genes (Appendix 4). Intriguingly, 20 of these genes were observed to be simultaneously affected by gains and losses. The gained genes were located in eight chromosomal regions, namely 1p22, 1q12-q32, 2p23.3, 3p24-p26, 6p21.3, 8q22-q24, 10q26 and 18p11.32 (Appendix 3), whereas the genes affected by losses mapped to 13 regions: 2q33.1, 4q13-q35, 6p21, 6q14.1, 7p15.2, 7q22.1, 13q11-q31, 14q32.3, 16q22-q24, 19p13, Xp22.3 and Xq26-q28 (Appendix 4).

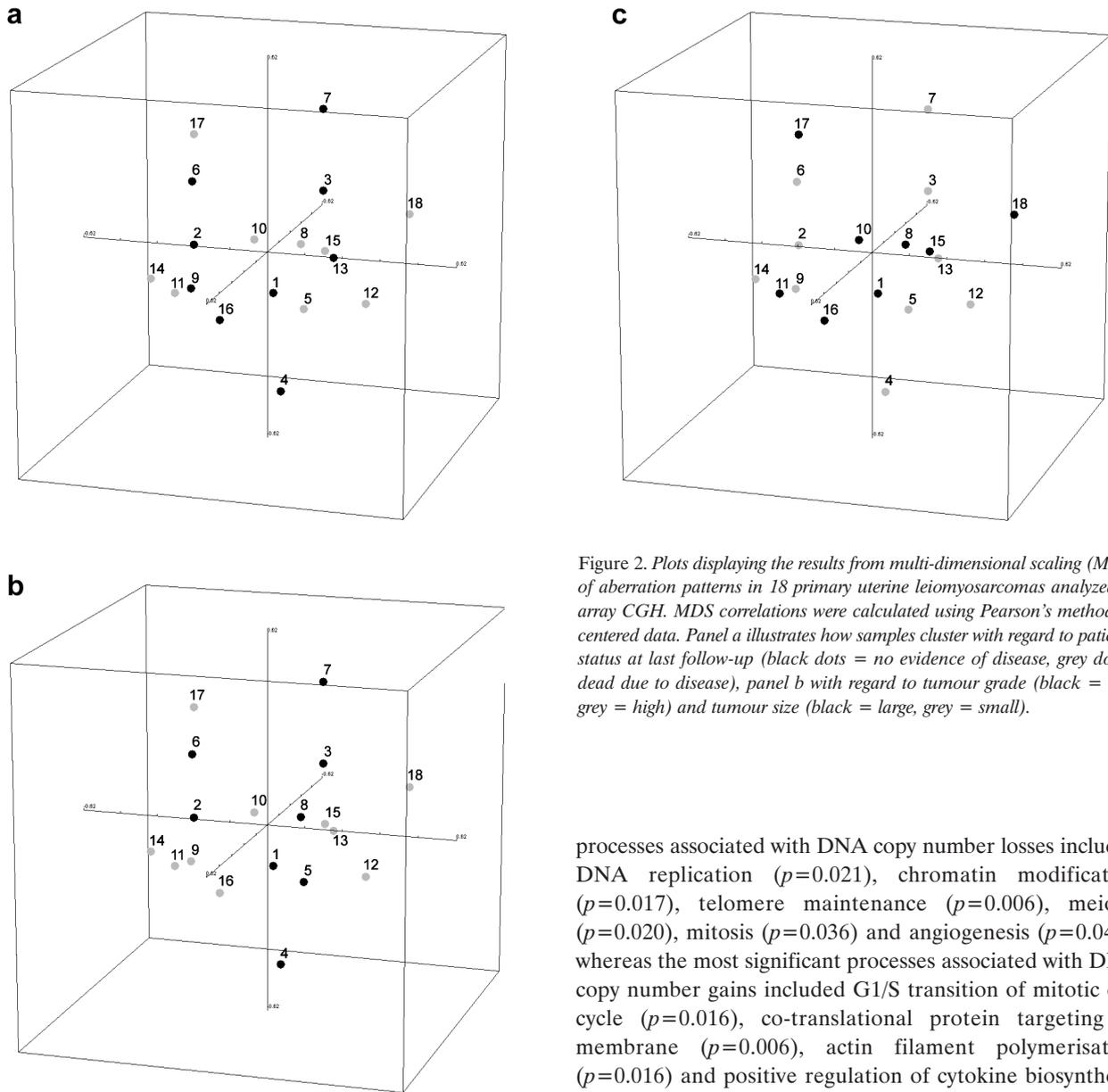


Figure 2. Plots displaying the results from multi-dimensional scaling (MDS) of aberration patterns in 18 primary uterine leiomyosarcomas analyzed by array CGH. MDS correlations were calculated using Pearson's method on centered data. Panel a illustrates how samples cluster with regard to patients' status at last follow-up (black dots = no evidence of disease, grey dot = dead due to disease), panel b with regard to tumour grade (black = low, grey = high) and tumour size (black = large, grey = small).

To uncover the biological networks underlying the observed aberration patterns detected by aCGH, the identified genes were classified according to the gene ontology classification system (16). We focussed on the most relevant categories described in the "biological process" ontology. In order to evaluate which of the identified biological processes were specifically targeted in ULMS, an enrichment analysis was performed. Applying separate sets of hypergeometric tests for the commonly gained and lost genes, we were able to pinpoint a number of processes that were statistically significantly enriched. Several interesting

processes associated with DNA copy number losses included DNA replication ($p=0.021$), chromatin modification ($p=0.017$), telomere maintenance ($p=0.006$), meiosis ($p=0.020$), mitosis ($p=0.036$) and angiogenesis ($p=0.040$), whereas the most significant processes associated with DNA copy number gains included G1/S transition of mitotic cell cycle ($p=0.016$), co-translational protein targeting to membrane ($p=0.006$), actin filament polymerisation ($p=0.016$) and positive regulation of cytokine biosynthesis ($p=0.018$) (Appendices 7 and 8). A graphical representation of all enriched GO categories and their relations in the ontology hierarchy are shown as directed acyclic graphs in Appendices 5 and 6.

Multidimensional scaling analysis was used to observe any associations between specific gene copy number changes and clinically relevant factors, namely tumour grade (low vs. high), tumour size (>10 cm vs. ≤ 10 cm) and status at last follow-up (death vs. no evidence of disease). Figure 2 shows the plots of sample similarities with regard to the three most significant dimensions related to the observed aberration patterns. Based on the apparent lack of data point clustering together with the clinical parameters, we concluded that the aberration patterns were not significantly affected by any of the parameters.

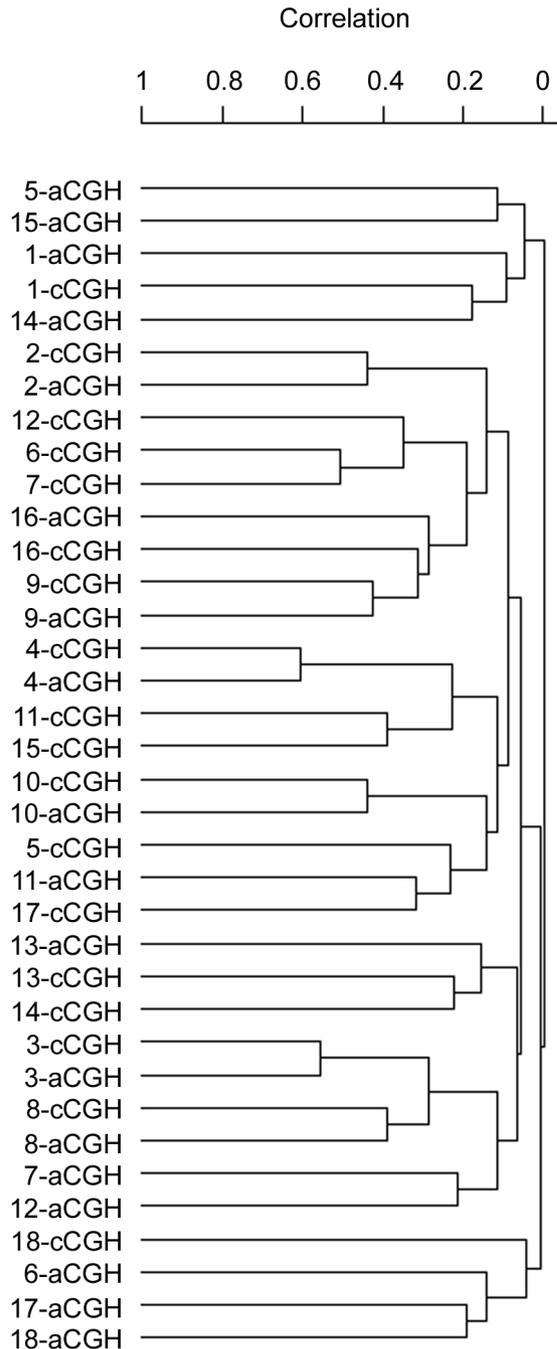


Figure 3. Correlation-based hierarchical clustering to compare the results obtained by conventional CGH and array CGH in 18 primary leiomyosarcoma samples. Sample similarity was assessed using Pearson's correlation and the tree-structure was obtained using average linkage amalgamation rules.

In order to make aCGH and cCGH results feasible for comparisons, the aCGH data was scaled down to meet the resolution of cCGH through an averaging procedure.

Hierarchical clustering of the 18 pairs of samples (Figure 3) clearly showed that there was no systematic methodological difference, because the root node did not segregate into sub-trees consisting only of aCGH or cCGH samples. However, in six cases (samples 2, 3, 4, 8, 9 and 10) the most similar sample pairs consisted of the aCGH sample and its cCGH counterpart. Furthermore, in three other cases (samples 1, 13 and 16) the matched pair was the second most similar, whereas in yet another case (sample 18) the match was the third highest correlated one.

Discussion

Array comparative genomic hybridisation (aCGH) analysis was performed on 18 primary uterine leiomyosarcomas (ULMS), the largest cohort of patients analysed by this technology so far. The aim of the study was to obtain a genome-wide pattern of DNA copy number changes and to uncover the biological networks involved in this tumour using the pathway analysis approach. Furthermore, we assessed possible associations between DNA copy number changes and tumour grade, tumour size or follow-up status of the patients.

All ULMS included in the study had previously been analysed by conventional CGH (cCGH) by Svarvar *et al.* (10). To resolve the complexity in the patterns of aberrations detected in all of the 18 samples, we discarded aberrations that were not common to at least 20% of cases, thereby focussing on the most representative changes and excluding spurious single aberration events. In an effort to elucidate the cellular mechanisms underlying the observed aberration patterns, we applied a strategy known as *pathway analysis*. Using annotation information provided by the gene ontology classification, we compiled all the biological processes to which the genes commonly affected by copy number changes could be ascribed. An analysis aimed at identifying those processes that were specifically targeted in ULMS was performed using the hypergeometric test to assess the degree of enrichment of genes in any particular process. By doing so, we not only aimed at gaining a biologically meaningful interpretation of the highly complex aberration patterns observed, but also at further limiting the possibility of spurious findings. As a result of this approach several cellular processes previously known to be implicated in the development and/or progression of cancer were identified among the genes affected by losses, *i.e.*, angiogenesis, telomere maintenance, mitotic and meiotic cell cycle, DNA replication and chromatin modification. In the present study, three genes, *EREG*, *FLT1* and *NOTCH4*, showed copy number losses and were involved in angiogenesis. The genes involved in telomere maintenance were *DKC1* and *TERF2IP*. The telomere maintenance activity is a hallmark of most

cancers. In our study two genes involved in telomere maintenance showed DNA copy number loss, which may indicate that senescence of the telomeres could be a delayed feature in ULMS leading to tumorigenesis. Because regulation of mitotic and meiotic cell cycle has an essential role in normal development and tumour prevention, copy number loss of the genes involved in both of these cell cycles may contribute to tumour growth and proliferation. Genes in both mitotic and meiotic cell cycle pathways were affected by DNA copy number loss in our ULMS cases. Genes lost in mitotic cell cycle were *BRCA2*, *CCNA1*, *CETN2*, *EREG* and *FZRI*, and in meiotic cell cycle *CCNA1*, *EREG* and *MSH5*. The loss of the genes in mitotic and meiotic cell cycle may lead to abundant proliferation of tumour cells. *EREG*, *HMGB1*, *MSH5* and *RFC3*, involved in DNA replication, showed copy number losses. In malignant fibrous histiocytoma *EREG* has been recognised as a growth factor that is involved in tumour development, and, therefore, considered an important tumour growth regulator (17). *HMGB1* functions in a number of biological processes including transcription and DNA repair, as coactivator of p53, stabilises nucleosomes, and allows bending of DNA that facilitates gene transcription. *HMGB1* has also been implicated as a possible target for cancer therapy (18). *MSH5* is known to have functional roles in many cellular processes, such as DNA damage response and meiotic homologous recombination, and the normal *MSH5* function is essential for meiotic progression (19). *RFC3* coordinates DNA synthesis and DNA repair. The genes that are involved in chromatin modification and were lost in our study were *BRCA2*, *DOTIL*, *HMG20B*, *RING* and *SIRT*. *BRCA2* is a tumour suppressor that is thought to be critical for replication and repair of DNA during the S phase (20). *DOTIL* is involved in epigenetic modifications by methylating nucleosomal histone H3. *HMG20B* is involved in the regulation of transcription and chromatin conformation, whereas *RING1* functions as a transcriptional repressor and deregulator of the expression levels of certain oncogenes (21).

Genes in the most intriguing biological processes affected by DNA copy number gains in our study were involved in G1/S transition of the mitotic cell cycle, co-translational protein targeting to the membrane, actin filament polymerisation and in positive regulation of cytokine biosynthesis. *CDC7* and *GFI1* are involved in G1/S transition of the mitotic cell cycle pathway. *CDC7* plays a major role in regulation of cell cycle progression and it is also required for the initiation of DNA replication, whereas *GFI1* is a proto-oncogene (22). Genes with copy number gains in the current study involved in actin filament polymerisation were *ARPC4* and *ARPC5*. Actin cytoskeleton is modified in transformed cells. Stournaras *et al.* (23) have shown that reduced amounts of polymerised actin are expressed by malignant cells. Zyxin, a potential regulator of

actin polymerisation acts as tumour suppressor in Ewing tumour cells (24), whereas in our ULMS the genes present in actin filament polymerisation were gained. In our study, two genes involved in the positive regulation of cytokine biosynthesis showed gains, *i.e.*, *APOA2* and *GLMN*. Amplification of *APOA2* has been found in various sarcomas (25), whereas *GLMN* affects differentiation of vascular smooth muscle cells and vascular morphogenesis, especially in cutaneous veins.

Genes involved in other biological processes in our ULMS, such as co-translational protein targeting to the membrane (*SSR2*, *SSR3*), have not been associated with carcinogenesis but they may offer new insights into the mechanisms of tumorigenesis.

Recently, Cho *et al.* reported an analysis of seven ULMS by aCGH with 1,440 BAC clones (11), but tumour grading and the method for data analysis are not compatible with our studies. The highly amplified BAC clones contained *HMGIC*, *SAS*, *MDM2* and *TIMI1*, whereas the clones that were frequently gained were *TIMI1*, *PDGFR-B*, *RECQ4*, *VAV2*, *FGF4*, *KLK2*, *PNUTL1*, *GDNF*, *FLG*, *EXT1*, *WISP1*, *HER-2* and *SOX18*, and the clones that were frequently lost were *LEU1*, *ERCC5*, *THBS1*, *DCC*, *MBD2*, *SCCA1*, *FVT1*, *CYB5* and *ETS2/E2*. Discrepancies between these findings and our present results could be explained by the different array platforms, different data analysis methods, resolution differences between BAC and cDNA arrays, and by larger number of samples in our study (18). In general, comparisons to other microarray studies are less informative due to wide technical differences between array platforms and differences in criteria applied to the identification of significant genes.

The *C-KIT* proto-oncogene (4q11-12) has previously been found to be expressed in ULMS (26, 27), which makes it a potential target for therapeutic treatment. Our aCGH showed no DNA copy number gains of this proto-oncogene. Because gene expression was not studied in our samples, we cannot construe whether an amplification step is required for *C-KIT* overexpression.

Assessment of possible associations between aCGH patterns and clinically relevant parameters did not provide any evidence for such association with regard to tumour grade, tumour size or patient status at last follow-up. Although similar findings have been reported by Mandahl *et al.* (28), it should be kept in mind that even when our ULMS sample set is so far the largest analysed by aCGH, 18 cases is still a rather limited number in statistical terms.

The aCGH and cCGH methodologies were found to be comparable as the pairwise correlation showed no evidence of systematic differences when the different resolution of both methods was controlled. Others have also juxtaposed aCGH and cCGH in several cancers (29-31). In pancreatic cancer, the amplifications identified by aCGH were approximately three-fold compared to cCGH. This demonstrates the power

of aCGH in detecting amplifications and enabling identification of novel target genes in tumorigenesis.

In conclusion, our results clearly showed that the array-based CGH technique provides a high-resolution mapping of chromosomal aberrations in ULMS. The majority of genes that were implicated in various cellular pathways are associated with tumour development and progression. However, we found no evidence suggesting correlation between gene copy number patterns and clinical parameters. Comparison between cCGH and aCGH results demonstrated that the results obtained using different technologies agree well when the inherent differences in resolution are controlled.

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