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Gene Expression Patterns and Gene Copy Number Changes in Dermatofibrosarcoma Protuberans

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Dermatofibrosarcoma protuberans (DFSP) is an aggressive spindle cell neoplasm. It is associated with the chromosomal translocation, t(17;22), which fuses the *COL1A1* and *PDGFβ* genes. We determined the characteristic gene expression profile of DFSP and characterized DNA copy number changes in DFSP by array-based comparative genomic hybridization (array CGH). Fresh frozen and formalin-fixed, paraffin-embedded samples of DFSP were analyzed by array CGH (four cases) and DNA microarray analysis of global gene expression (nine cases). The nine DFSPs were readily distinguished from 27 other diverse soft tissue tumors based on their gene expression patterns. Genes characteristically expressed in the DFSPs included *PDGFβ* and its receptor, *PDGFRB*, *APOD*, *MEOX1*, *PLA2R*, and *PRKCA*. Array CGH of DNA extracted either from frozen tumor samples or from paraffin blocks yielded equivalent results. Large areas of chromosomes 17q and 22q, bounded by *COL1A1* and *PDGFβ*, respectively, were amplified in DFSP. Expression of genes in the amplified regions was significantly elevated. Our data shows that: 1) DFSP has a distinctive gene expression profile; 2) array CGH can be applied successfully to frozen or formalin-fixed, paraffin-embedded tumor samples; 3) a characteristic amplification of sequences from chromosomes 17q and 22q, demarcated by the *COL1A1* and *PDGFβ* genes, respectively, was associated with elevated expression of the amplified genes. *(Am J Pathol 2003, 163:2383–2395)*

Dermatofibrosarcoma protuberans (DFSP) is an uncommon soft tissue neoplasm of young adults with a propensity for locally aggressive growth. This tumor typically arises in the subcutaneous tissues of the trunk and proximal extremities and is composed of uniform spindle cells of uncertain histogenesis. Most DFSPs are cured by wide surgical excision, but local recurrence occurs in approximately 20% of cases.[1] In some instances, radiotherapy can be of benefit.[2] Death due to metastatic disease is very rare (<5%),[1] and there have been isolated reports of apparently successful treatments for metastatic disease with imatinib.[3,4] Virtually all cases of DFSP have a translocation that involves chromosomes 17 and 22, resulting in fusion of the collagen type I α I (*COL1A1*) and platelet-derived growth factor β (*PDGFβ*) genes.[5,6] The regulatory sequences of *COL1A1* lead to increased expression of a fusion transcript that is processed to wild-type *PDGFβ*, which has transforming activity when expressed at high levels.[7,8] The translocation is most commonly located on a ring chromosome with varying amounts of DNA derived from chromosomes 17 and 22, and sometimes also from other chromosomes,[9] associated with amplification. To date, the areas of amplification have only been crudely mapped using conventional genomic hybridization.[10–12] Fusion of *COL1A1* and *PDGFβ* is also encountered in giant cell fibroblastoma, a rare tumor typically seen in children. Currently these two tumors are thought to be variants of the same lesion.[5,6]

Expression profiling using cDNA microarrays has resulted in molecular subclassification of a wide variety of tumors, including lymphomas,[13] lung carcinomas,[14] and breast carcinomas.[15] Gene expression profiling with cDNA microarrays has also proven useful as an aid in the differential diagnosis of mesenchymal tumors, where morphological features show significant overlap.[16–20] cDNA microarrays have been used not only for gene expression studies but also for high-resolution comparative genomic hybridization (array CGH). By using the

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same microarrays for both array CGH and cDNA expression studies on the same tumor samples, Pollack et al[21] found that changes in gene copy number were consistently associated with corresponding changes in expression of the affected genes. In the case of breast carcinoma, the majority of amplified genes showed increased expression, and alterations of gene copy number accounted for a significant component of the altered gene expression observed in an individual tumor.

In this study we used DNA microarrays to profile gene expression in DFSP and identified a large number of genes with consistently high expression in these tumors. Several potential differential diagnostic markers were identified, one of which (*APOD*) is described in detail in a separate report (West et al, in preparation). In addition, we characterized DNA copy number alterations in DFSP at a high resolution and correlated those changes with expression levels of affected genes. In the course of this study we found that array CGH could be used successfully to analyze formalin-fixed, paraffin-embedded tissue, circumventing the need for fresh frozen tissue, and making possible array CGH studies on samples from surgical pathology archives.

Materials and Methods

Case Selection

Nine cases of DFSP were used for this study, obtained from the Departments of Pathology of Stanford University Medical Center, Vancouver Hospital and Health Sciences Centre, University of Washington Medical Center, and Cleveland Clinic Foundation. Fresh frozen tissue, stored at −80°C was available in each case. The Institutional Review Board at Stanford University Medical Center approved the study. The diagnosis of DFSP was based on the light microscopic appearance of the tumors, with confirmatory immunohistochemical staining (CD34) performed in each case. All but one of the DFSPs reacted for CD34, consistent with known staining patterns. For gene expression studies, a total of 36 soft tissue tumors (STT) were studied, including the nine cases of DFSP. Non-DFSPs studied were leiomyosarcoma (eight cases), malignant fibrous histiocytoma (six cases), of which two cases could be subclassified as myxofibrosarcoma, gastrointestinal stromal tumor (GIST, five cases), synovial sarcoma (six cases), nodular fasciitis (one case), and epithelioid fibrous histiocytoma (one case). One of the GIST cases (STT1823) was a recurrence from a prior lesion (STT094). The nine DFSP cases, the two myxofibrosarcomas, the nodular fasciitis, the fibrous histiocytoma, and the recurrence of the GIST lesion are new to this report. The other lesions functioned as comparison for gene expression level determination in DFSP and were previously published as part of a prior study.[16]

Array CGH studies were performed using fresh frozen tissue on four of the nine DFSP cases, and also using paraffin embedded tissue for three of the four cases. For comparison, we performed array CGH on four cases of solitary fibrous tumor (SFT), a soft tissue tumor known to have a relatively simple karyotype[22] that rarely harbors chromosome abnormalities in the 17q or 22q regions.[23] DNA was also isolated from paraffin- embedded material of six fibrous histiocytoma specimens for aCGH studies. In each case the diagnosis was made based on conventional light microscopic examination, with appropriate adjuvant immunohistochemical studies.

Histology and Immunohistochemistry

All cases were reviewed by two pathologists (B.R. and M.v.d.R.). For immunohistochemistry, sections were stained with CD34 (Becton Dickinson, Mountain View, CA) and APOD (Novocastra, Newcastle-on-Tyne, United Kingdom) using the Envision detection system (DAKO, Carpinteria, CA).

cDNA Expression Microarray Analysis

The cDNA microarrays used in this study included about 28,000 unique characterized genes or ESTs represented by a total of 41,859 unique cDNAs printed on glass slides by the Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/jsp/home.jsp). The details of the construction of these arrays were described previously.[15] Preparation of tumor mRNA, labeling, and hybridization were performed as described in an earlier publication.[15] Briefly, after confirmation of the presence of viable tumor by frozen section, tissue was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was prepared; mRNA was then isolated using the FastTrack 2.0 method following the manufacturer's protocol. Preparation of Cy-3-dUTP (green fluorescent) labeled cDNA from 2 μg of reference mRNA and Cy-5-dUTP (red fluorescent) labeled cDNA from 2 μg of each tumor specimen mRNA, microarray hybridization and subsequent analysis was performed as described.[15] The reference mRNA was isolated from a pool of 11 cell lines.[15] After washing, the microarrays were scanned on a GenePix 4000 microarray scanner (Axon Instruments, Foster City, CA) and, after normalization of fluorescence intensities to control for experimental variation, fluorescence ratios (tumor/reference) were calculated using GenePix software. The primary data tables and the image files are freely available from the Stanford Microarray Database[24] (http://genome-www4.stanford.edu/MicroArray/SMD/). Data were selected using the following criteria: 1) only cDNA spots with a ratio of signal over background of at least 1.5 in either the Cy3 or Cy5 channel were included; 2) genes were included for further analysis only if the corresponding cDNA spots provided data that passed criterion 1 on at least 29 of 36 arrays (80% good data); 3) to focus on genes with high variations in expression in these tumors, we selected genes whose expression level differed by at least fourfold in at least two specimens from that gene's geometric mean expression level across all 36 specimens. Hierarchical clustering analysis[25] and significance analysis of microarrays (SAM)[26] were then performed as described previously.[16]

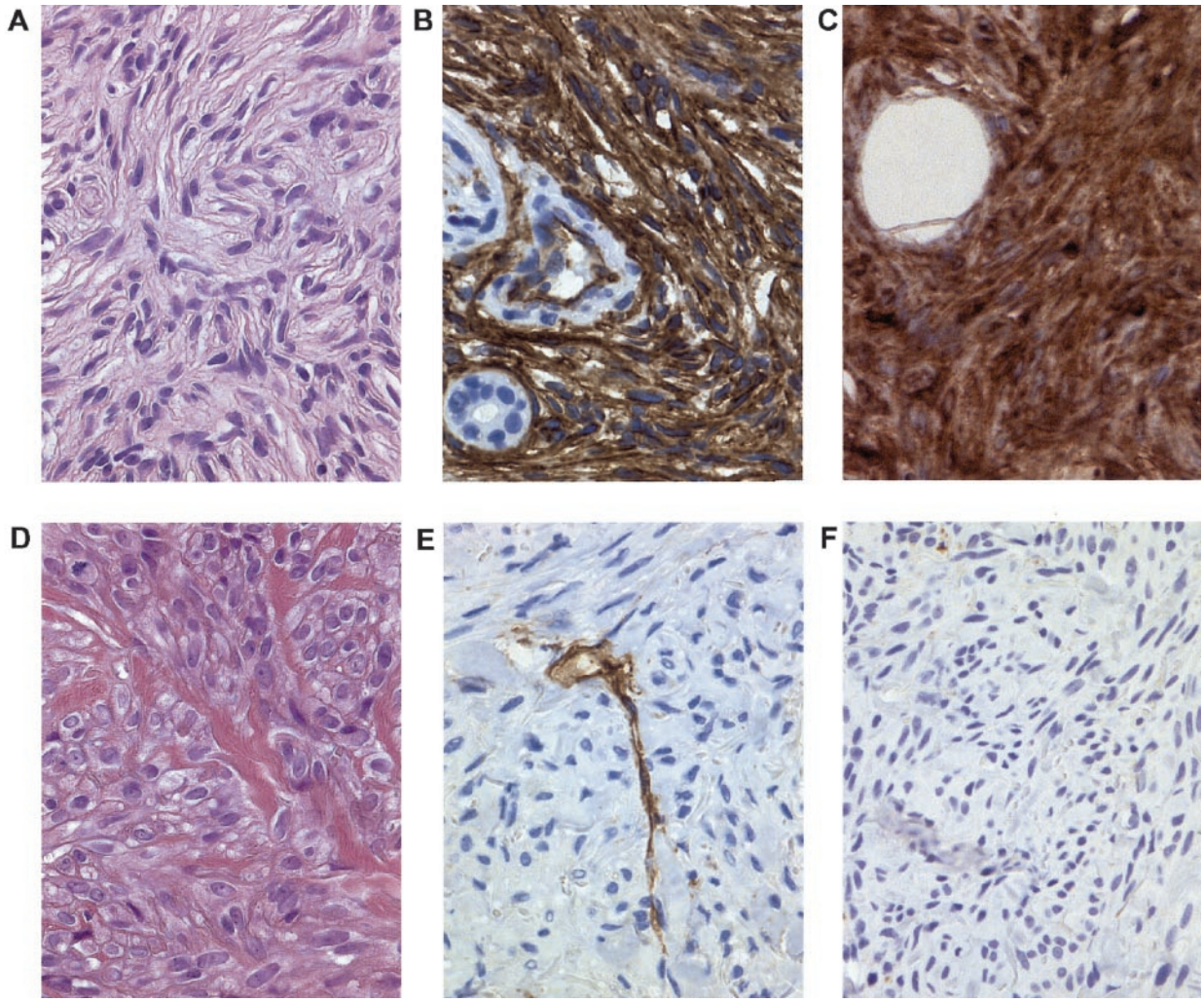


Figure 1. Hematoxylin and eosin staining of a representative case of DFSP (STT491) (**A**), and a cellular fibrous histiocytoma (STT169) (**D**), with confirmatory immunohistochemical staining for CD34 (**B** and **E**), and APOD (**C** and **F**). The DFSP is positive for CD34 (**B**), while the cellular fibrous histiocytoma (**E**) shows CD34 positivity in endothelial cells only, but not in tumor cells. IHC for APOD is strongly positive in DFSP (**C**), while the FH fails to stain with antibodies against APOD (**F**) despite relative high levels of *APOD* mRNA.

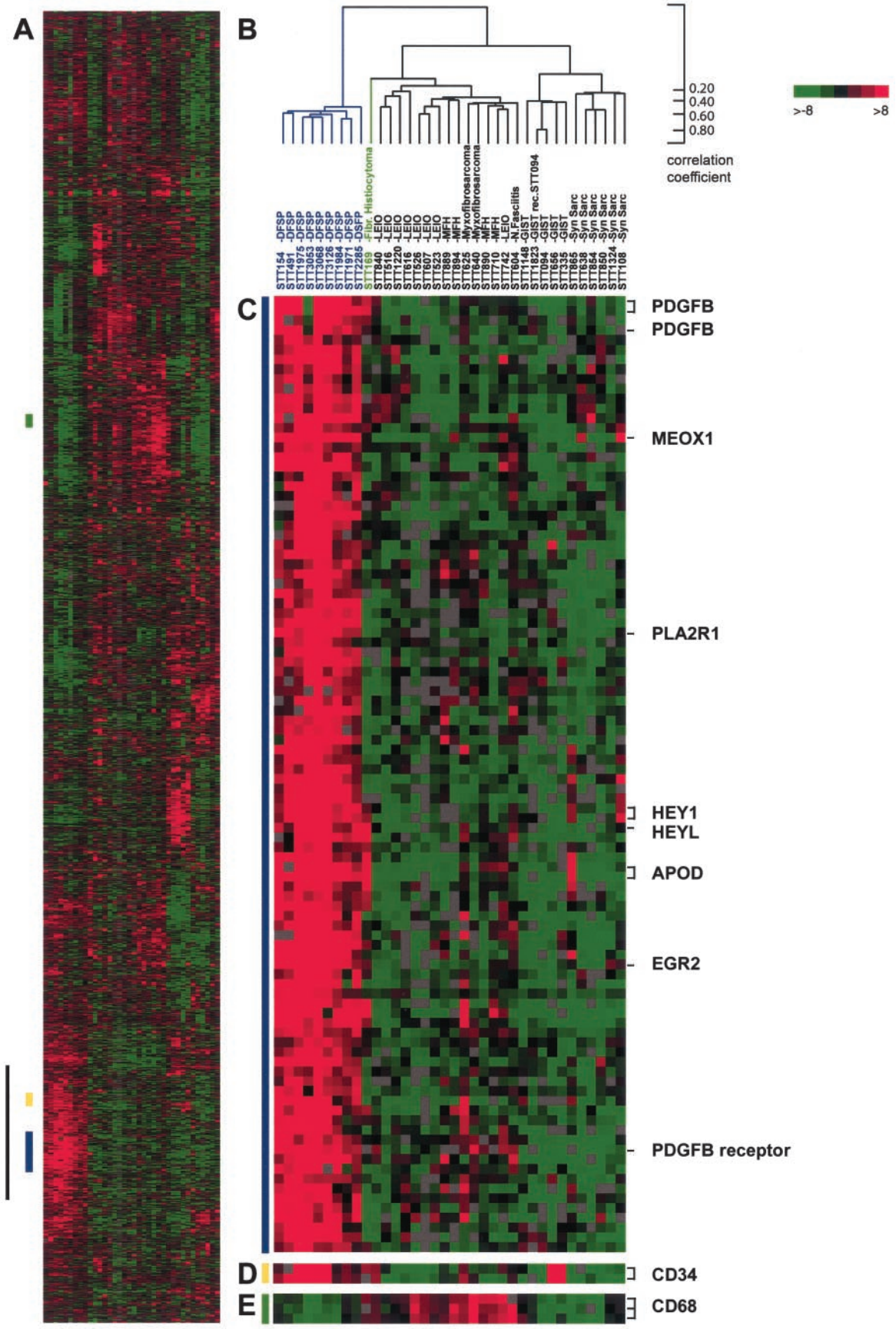
Array Comparative Genomic Hybridization

Of the over 41,000 cDNA sequences represented on the microarrays used for this study, the chromosomal localization is known for 35,151 distinct mapped cDNAs, which represent 24,540 different Unigene clusters and 3225 cDNAs not yet represented in Unigene clusters. Tumor DNA from frozen or formalin-fixed, paraffin-embedded

Table 1. Clinical Data of All DFSP Cases and of the Four DFSP Mimics

	Age	Sex	Site	CD34	APOD
STT154-DFSP	46	F	Scapula	+	+
STT491-DFSP	41	F	Sacrum	+	+
STT1975-DFSP	60	M	Rt shoulder	+	+
STT3053-DFSP	49	M	Rt shoulder	+	+
STT3068-DFSP	48	F	Central back	+	+
STT3126-DFSP	46	F	Lt breast	+	+
STT1984-DFSP	32	F	Abd wall	+	+
STT1971-DFSP	37	F	Lt ant abd wall	−	+
STT2285-DSFP	37	M	Rt thigh	+	+
STT 604-NF	50	F	Submandibular	−	−
STT 625-myxofibrosarc	65	F	Chest	−	−
STT 640-myxofibrosarc	67	F	Rt arm	−	−
STT 169-FH	72	F	Rt arm	−	−

F, female; M, male; Rt, right; Lt, left; Abd, abdominal; ant, anterior.


A
B
0.20
0.40
0.60
0.80
correlation coefficient
>-8
>8
STT154 -DFSP
STT1491 -DFSP
STT1975 -DFSP
STT3063 -DFSP
STT3126 -DFSP
STT1984 -DFSP
STT1971 -DFSP
STT2285 -DSFP
STT169 -Fibr. Histiocytoma
STT840 -LEIO
STT516 -LEIO
STT1220 -LEIO
STT1616 -LEIO
STT1626 -LEIO
STT1607 -LEIO
STT523 -LEIO
STT889 -MFH
STT894 -MFH
STT625 -Myxofibrosarcoma
STT640 -Myxofibrosarcoma
STT890 -MFH
STT710 -MFH
STT742 -LEIO
STT604 -N.Fasciitis
STT1143 -GIST
STT1824 -GIST rec.STT094
STT094 -GIST
STT656 -GIST
STT335 -GIST
STT865 -Syn Sarc
STT638 -Syn Sarc
STT854 -Syn Sarc
STT850 -Syn Sarc
STT1324 -Syn Sarc
STT108 -Syn Sarc
C
PDGFB
PDGFB
MEOX1
PLA2R1
HEY1
HEYL
APOD
EGR2
PDGFB receptor
D
CD34
E
CD68

tissue and reference DNA (normal gender-matched human leukocytes) were extracted; see protocols on accompanying website (http://genome-www.stanford.edu/DFSP/). Frozen tumor DNA and reference DNA were digested with *Dpn*II before further processing. Gel electrophoresis of digested and non-digested DNA isolated from formalin-fixed, paraffin-embedded tissue was run to determine DNA fragment size. Labeling of DNA isolated from tumor samples, after light-microscopic confirmation of the presence of non-necrotic tumor, was performed as described previously[21] (http://cmgm.stanford.edu/pbrown/protocols/index.html). Briefly, 2 to 4 μg of tumor DNA was fluorescently labeled (Cy5) in a volume of 50 microliters, mixed with reference DNA labeled with Cy3, and hybridized overnight to the array. After washing, the array slides were scanned on a GenePix Scanner (Axon Instruments) and fluorescence ratios (test/control) calculated using GenePix software. Only cDNA spots with a ratio of signal over background of at least 1.5 in the Cy3 channel were included in further analysis. Chromosomal localization of the mapped genes was assigned as described previously[21] and is based on Goldenpath data from June 28, 2002. For CGH data the copy number for each locus was based on a moving average of the five nearest cDNA clones centered on that locus.[21] For chromosome 17 and 22, 1224 and 527 genes were selected respectively, and the same genes were used to display centered expression levels for four DFSPs and three SFTs in Figure 4.

Statistical Analysis

For statistical tests the SPSS version 10.0 statistical software package (SPSS, Inc., Chicago, IL) was used.

Results

Histology and Immunohistochemistry

The nine cases of DFSP studied here were all typical DFSP based on their histological features and/or immunohistochemical staining profiles (Figure 1). Clinical and immunohistochemical features of the DFSPs are shown in Table 1. The 27 cases of non-DFSP tumors chosen for comparison by gene expression profiling included four tumors with histological features similar to those of DFSP. One, STT169, (Figure 1) was a cellular fibrous histiocytoma, the lesion with which DFSP is most often confused. The distinction between these two neoplasms is critical, as histiocytomas are almost invariably benign and require less aggressive treatment. Three other lesions, two myxofibrosarcomas (STT625, STT640) and one case of nodular fasciitis (STT604) can be confused histologically with the myxoid variant of DFSP. The clinical and immunohistochemical features of the four DFSP mimics are also shown in Table 1. The histology of the nine DFSPs, and the four lesions in its differential diagnosis can be found on the supplemental Figure 1 (http://genome-www.stanford.edu/DFSP/). The histology of the remaining 23 STT can be found on the supplemental website of an earlier publication (http://genome-www.stanford.edu/sarcoma/).

Gene Expression Data

This report focuses on variation among these tumors in expression of 4687 of the 28,597 genes or ESTs represented on our microarrays. These were genes for which we consistently were able to obtain technically adequate measurements and where expression levels varied substantially among the 36 samples analyzed (Figure 2A). We first used an unsupervised hierarchical clustering method to highlight groups of tumors with similarities in global gene expression patterns. Hierarchical clustering analysis organizes genes into groups with similar expression patterns, facilitating recognition of functional themes in the expression patterns, and similarly organizes the soft tissue neoplasms into distinct groups. The results are displayed in Figure 2A in the form of a table in which the expression pattern of each gene in each tumor is represented using a color key and dendrograms are used to represent relationship among the tumors. The nine cases of DFSP clustered closely together based on their global gene expression patterns, indicating that they were closely related to each other and significantly different from the other tumors profiled (Figure 2B). The 4 cases representing alternative diagnoses in the differential diagnosis of DFSP (STT640, STT625, STT169, STT604) clustered separately from the cases of DFSP.

DFSP specimens were distinguished from the other neoplasms by a large cluster of 465 genes whose patterns of variation in expression among these tumors were highly correlated (correlation coefficient with groupwise mean pattern of $>$0.63, see black bar, Figure 2A). Within this cluster is a subcluster of 98 genes including the *PDGFβ* and *PDGFRB* genes, shown in Figure 2C. The complete dataset is available in a searchable format on the accompanying website (http://genome-www.stanford.edu/DFSP/), where the 465 genes are also listed as Web Table 2. The DFSPs expressed high levels of *PDGFβ* transcripts, consistent with the known significance for this

Figure 2. Hierarchical cluster analysis of gene expression profiles of 36 tumors, including nine DFSPs. Each **row** represents the relative level of expression for a single gene, centered at the geometric mean of its expression level across the 36 samples. Each **column** shows the expression levels for a single sample. The red or green colors indicate high or low expression, respectively, relative to the mean. The intensity of the colors indicates the magnitude of deviation from the mean (see color bar). Gray denotes missing data. **A:** Overview of expression pattern of the 4687 genes used for hierarchical clustering analysis. The black vertical bar highlights a cluster of 465 genes that are characteristically expressed in DFSP. The yellow, green, and blue bars indicate the areas shown in more detail in **C**, **D**, and **E**. **B:** The same clustering analysis shown in **A** but with only a subset of the gene expression data displayed, consisting of genes most characteristic of the DFSPs. At the top is the dendrogram depicting the relatedness between tumor samples, with short branches denoting a high degree of relatedness (see correlation coefficient bar). DFSP is shown in blue. Leio, leiomyosarcoma; MFH, malignant fibrous histiocytoma; N.Fasciitis, nodular fasciitis; Fibr. Histiocytoma, cellular fibrous histiocytoma (in green); Synsarc, synovial sarcoma; GIST, gastrointestinal stromal tumor. Gene names are shown on the right. **C:** Relative mRNA levels in the 36 tumors for a set of 98 genes (correlation coefficient 0.82) that clustered with the *PDGFβ* gene. **D:** Expression level of *CD34*, measured by two separate spots on the arrays. **E:** Expression level of *CD68*, measured by three separate spots on the arrays.

tumor of the t(17;22) involving *PDGFβ*. Only one of the nine DFSPs, STT3053, did not show an elevated level of *PDGFβ* expression. The lack of *PDGFβ* mRNA expression as seen by gene array analysis in this case (using two separate cDNAs for *PDGFβ* mRNA) was confirmed by *in situ* hybridization studies (data not shown). Despite the lack of elevated *PDGFβ* expression in case STT3053 this is still considered a classic example of DFSP, based on histology and immunohistochemistry. The histology is available on the accompanying website and the tumor reacted for CD34 (see Table 1). The expression pattern of several thousands of genes, shared by case STT3053 and the other DFSP cases further supports this. Importantly, the receptor for PDGFβ, *PDGFRB*, was also highly expressed in the group of DFSPs. High levels of CD34 transcripts and low expression of *CD68* in DFSPs compared to non-DFSPs, are consistent with the known immunohistochemical profile of these tumors (Figure 2, D and E).[27–29] A cellular fibrous histiocytoma (case STT169) unexpectedly showed moderately high levels of *CD34* mRNA. However, immunohistochemical staining of this tumor shows that CD34 protein is expressed by endothelial cells, rather than the tumor cells themselves (Figure 1E). Although *APOD* mRNA was highly expressed in both DFSPs and the cellular fibrous histiocytoma (STT169), immunohistochemistry for APOD protein showed expression only in the DFSPs but not in the histiocytoma (Figure 1F). The full explanation for this discrepancy is unknown. However posttranslational modifications such as glycosylation may account for this effect. In addition in several instances, discrepancies between mRNA levels and protein levels have been described, for example in *bcl2* expression.[30]

We used a supervised analytical method, SAM (Significance Analysis of Microarrays), as an alternative way to search for differentially expressed genes that distinguish DFSP from the other sarcomas.[26] A list of the 259 genes (288 cDNAs) most consistently expressed at higher levels in DFSP, as determined by SAM, is available on the accompanying website (supplementary Table 2, http://genome-www.stanford.edu/DFSP/) with their chromosomal location. The top 50 genes, most responsible for the distinction between DFSP and the other soft tissue lesions, is shown in Table 2. These genes representing candidate diagnostic markers for DFSP, include *APOD*, which stained all nine DFSPs in this study and failed to react with the single histiocytoma (despite high levels of mRNA in that lesion). The utility of APOD as an immunohistochemical marker is described in detail in a separate report (West et al, in preparation).

Array Comparative Genomic Hybridization

Array CGH analysis of four DFSP cases revealed abnormalities of chromosome arm 17q and 22q in all cases (Figure 3), corresponding to the most commonly identified cytogenetic abnormality in DFSP, a ring chromosome containing portions of chromosomes 17q and 22q, r(17:22).[23] This ring chromosome results in fusion of the COL1A1 and PDGFβ genes. In contrast, 4 cases of solitary fibrous tumors, showed no significant areas of amplification or deletion. The areas of amplification of chromosomes 17 and 22 in DFSP start at the *PDGFβ* and *collagen 1A1* genes, such that the *PDGFβ* and *collagen 1A1* constitute one of two borders in a contiguous amplified segment of 22q, and 17q respectively, (Figure 3, Panel A, B). One or more additional genetic abnormalities were identified in every case and gains and losses at some loci were seen in more than one case (see supplemental Figure 3). For instance, we observed recurrent gains of chromosome 5 (STT1971, STT1984), chromosome 7p (STT154, STT1971), chromosome 8 (STT154, STT1971, STT1984), chromosome 18 (STT1971, STT1984), and chromosome 21 (STT1971, 1984). In addition, loss of large parts of chromosome 19 was observed in STT1971 and STT1984. Many of the apparent losses and gains affected small segments of chromosomes and would not be expected to be detectable cytogenetically or by conventional comparative genomic hybridization.

Array CGH Analysis of Frozen versus *Formalin-Fixed, Paraffin-Embedded Tissue*

To test the feasibility of array CGH analysis of formalin-fixed, paraffin-embedded tumor samples we analyzed both fresh frozen and formalin-fixed, paraffin-embedded material from the same tumor, in three cases of DFSP. In each case the essential results were very similar (Figure 3). In an attempt to demonstrate the potential clinical usefulness of aCGH in the differential diagnosis of other lesions, we performed aCGH on five cases of fibrous histiocytoma, for which only paraffin material was available. All cases showed a lack of significant areas of gene amplification by this technique, including in the areas of chromosome 17 and 22 affected in DFSP (Figure 5 and accompanying website).

Correlation of Array CGH and Gene Expression Data

Comparison of array CGH and gene expression data could be done in four cases of DFSP. For these cases the copy number and gene expression were compared for the 27,765 genes for which the chromosomal location has been mapped. A correlation was observed between increased gene copy number and increased gene expression. This influence of DNA copy number on gene expression is evident in the colored map representations of DNA copy number and mRNA levels for 1224 genes on chromosome 17 and 527 genes on chromosome 22 (Figure 4). From visually inspecting Figure 4 it appears that there is a considerable difference in levels of expression between the regions of chromosomes 17 and 22, bounded by the *COL1A1* and *PDGFβ* genes, respectively. To test this observation more formally, using the difference in average mRNA levels between the DFSP and SFT specimens, we considered a change-point mod-

Table 2. First 50 Genes with Highest Differential Expression in DFSP According to SAM Analysis

Gene symbol	Gene name	UniGene #	GenBank #	SAM score(d)
ESTs		Hs.32345	AI668612	11.1886
EGR2	Early growth response 2	Hs.1395	AA446027	9.893322
PLA2R1	Phospholipase A2 receptor 1, 180 kD	Hs.171945	W04525	9.403624
PCDH18	Protocadherin 18	Hs.97266	AA704401	9.161416
ESTs	ESTs	Hs.192262	AI192496	8.982065
LOC51136	LOC51136 PTD016 protein	Hs.30154	N51514	8.894226
LFNG	Lunatic fringe homolog	Hs.159142	R56561	8.826694
OMD	Osteomodulin	Hs.94070	N32201	8.450854
CHRFAM7A	CHRNA7 and FAM7A fusion	Hs.353211	W93369	8.396351
PDGFβ	Platelet-derived growth factor β polypeptide	Hs.1976	W72000	8.303292
ESTs		Hs.348762	AA700772	8.011256
ESTs		Hs.323780	N22836	7.949673
KIAA1211	KIAA1211 protein	Hs.205293	N35889	7.908669
	Homo sapiens cDNA FLJ12815 fis, clone NT2RP2002546	Hs.49476	AA284237	7.765829
ARVCF	Armadillo repeat gene deletes in velocardiofacial syndrome	Hs.171900	H17198	7.637739
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	Hs.75929	AA136983	7.589666
CEP4	Cdc42 effector protein 4; binder of Rho GTPases 4	Hs.3903	AA449061	7.490882
MEOX1	Mesenchyme homeo box 1	Hs.438	AA426311	7.461304
APOD	Apolipoprotein D	Hs.75736	H15842	7.457497
PLG	Plasminogen	Hs.75576	T73090	7.127251
FZD7	Frizzled homolog 7	Hs.173859	N69049	7.07609
	Homo sapiens, clone IMAGE:3450973, mRNA	Hs.7921	AA620437	7.042933
SPRY2	Sprouty homolog 2	Hs.18676	AA453759	7.038161
ESTs		Hs.355694	AA778653	6.995952
ESTs		Hs.164680	AA463229	6.980936
POPX1	Partner of PIX 1	Hs.166351	W40125	6.959919
MS12	Musashi homolog 2	Hs.173179	N45139	6.9322
ABCA9	ATP-binding cassette, sub-family A (ABC1), member 9	Hs.301496	AI822061	6.920054
ESTs		Hs.268874	R98003	6.890625
ELLS1	Hypothetical protein Ells1	Hs.7913	N29882	6.860183
KIAA0638	KIAA0638 protein	Hs.77864	AA405486	6.84359
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	Hs.15780	AA099357	6.838837
SEPT6	Septin 6	Hs.90998	AA868745	6.75616
ESTs		Hs.50162	N67355	6.715135
SPAG9	Sperm-associated antigen 9	Hs.129872	AA399253	6.580305
KIAA0937	KIAA0937 protein	Hs.62264	AA487527	6.579918
	Homo sapiens clone 24628 mRNA sequence	Hs.129997	AA885845	6.569535
KLF5	Kruppel-like factor 5 (intestinal)	Hs.84728	R27011	6.539115
IL11RA	Interleukin 11 receptor, α	Hs.64310	AI822086	6.526972
FLJ90754	Homo sapiens mRNA full-length insert cDNA clone	Hs.8963	AA424560	6.520919
ACVRL1	Activin A receptor type II-like 1	Hs.172670	H42572	6.520158
PTGFRN	Prostaglandin F2 receptor negative regulator	Hs.300591	T83630	6.477428
ESTs		Hs.247150	AA459734	6.474894
DHRS6	Oxidoreductase UCPA	Hs.124696	R97516	6.466339
TTYH2	Tweety homolog 2	Hs.27935	AA434395	6.45494
SCDGF-B	Spinal cord-derived growth factor-B	Hs.112885	AA904948	6.433207
DACT1	Heptacellular carcinoma novel gene-3 protein	Hs.48950	AA487274	6.426798
GPM6B	Glycoprotein M6B	Hs.5422	N59368	6.411646
LZTS1	Leucine zipper, putative tumor suppressor 1	Hs.93605	H09757	6.3579
NRXN3	Neurexin 3	Hs.247837	AA450335	6.285427

Input parameters for SAM analysis: imputation engine is "10-nearest neighbor imputer"; data type is "two class, unpaired data"; data are in log scale; number of permutations is 1000; No blocked permutations; RNG seed is 1234567; (delta, fold change) is (2.46002); (upper cutoff, lower cutoff) is (4.02194, −6.08331). Computed quantities are: computed exchangeability factor S0 is 236.1422; S0 percentile is 0; false significant number (median, 90%) is (0.57990, 0.57990); false discovery rate (median, 90%) is (0.19928, 0.19928); Pi0Hat is 0.5799.

el[31] with a single change in average expression level occurring on chromosome 17 and another change on chromosome 22. The maximum likelihood estimate for the change-point on chromosome 17, based solely on gene expression measurements, was the gene *PHB* (prohibitin Hs.75323, AA055656, chromosome 17, start 47192492), 12 mapped genes toward the p terminus from *COL1A1* (Hs.172928, R48843, chromosome 17, start 47864928). On chromosome 22, the maximum likelihood estimate of the change-point was *MGAT3* (mannosyl (β-1,4-)-glycoprotein β-1,4-N-acetylglucosaminyltransferase Hs.348978, AA421473, chromosome 22, start 36501033), five mapped genes toward the q terminus from *PDGFβ* (Hs.1976, W72000, chromosome 22, start 36234216). A 95% confidence region for the location of the change-point on chromosome 17 barely excluded the COL1A1 gene; a 95% confidence region for the location of the change-point on chromosome 22 included the *PDGFβ* gene. Despite the significant correlation between amplification levels and expression levels it is clear that there remain many examples of amplified genes that are not overexpressed.

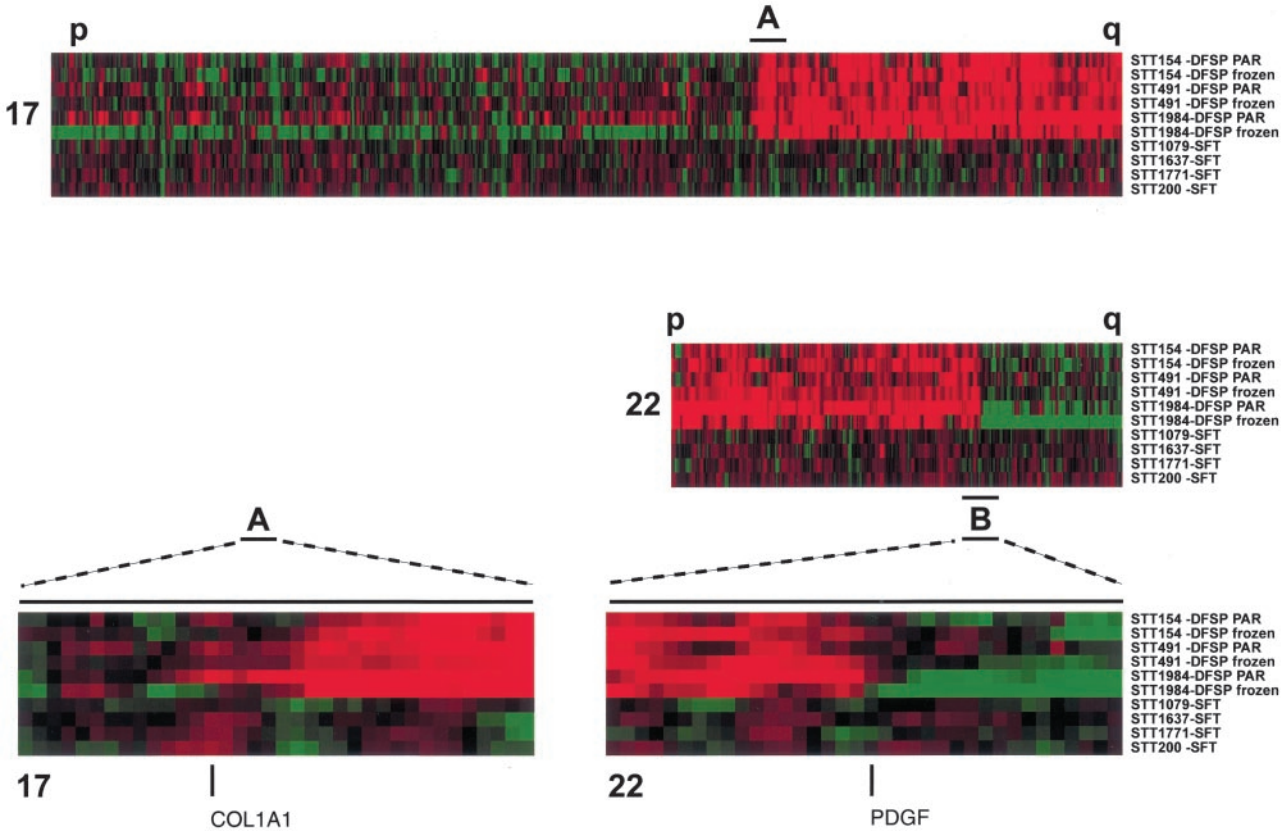


Figure 3. Pairwise comparison of array CGH on three cases of DFSP with DNA isolated from fresh frozen tumor and from formalin-fixed, paraffin-embedded tumor showing nearly identical regions of amplification on chromosomes 17 and 22. In contrast, five cases of solitary fibrous tumor show no significant changes on these chromosomes. Genes are arranged in order along the chromosome. Red indicates gene amplification, green indicates gene deletion. **A:** Magnified view of start of amplification region on chromosome 17. Note that this area starts at the location of *COL1A1*, one of the fusion partners in the t(17:22). **B:** Magnified view of start of amplification region on chromosome 22, starting at the location of *PDGFβ*, the other fusion partner in the t(17:22).

Of the 259 genes whose elevated expression was most consistently associated with a diagnosis of DFSP, 12% (31 genes) were localized to the regions of recurrent amplification on chromosomes 17 (23 genes) and 22 (8 genes), as determined by array CGH (Table 3), although the amplified segments of chromosomes 17 and 22 contain only 3% of all mapped genes ($P < 0.001$, Binomial test-exact). Although genes in regions of amplification tended to be more highly expressed, many of the genes that distinguish DFSP from other tumors are in regions without apparent changes in DNA copy number.

The full data sets for the array CGH and gene expression microarray studies can be accessed through the Stanford Microarray Database website and through http://genome-www.stanford.edu/DFSP/.

Discussion

Dermatofibrosarcoma protuberans was first described in 1924, but the cell of origin for this lesion remains unknown, and cases of DFSP continue to pose difficulties in differential diagnosis.[32] The uncertainties result from the tumor's histology, consisting of bland spindle cells that lack distinctive growth or cytological features, and the lack of specific immunohistochemical markers for this tumor. The correct diagnosis is critical, as DFSPs are locally aggressive tumors that need a wide surgical excision, whereas other lesions in the differential diagnosis of DFSP, such as fibrous histiocytoma, can be treated less aggressively. The local relapse rate of DFSP is around 20%. Relapses are often due to incomplete surgical resection.[1]

Gene expression profiling has been applied to a wide range of tumors and has repeatedly been successful in recapitulating established histological classifications,[13–16] and in identifying clinically relevant new subtypes of tumors, when routine diagnostic approaches have failed to do so.[13–15,33–38]

In the case of mesenchymal tumors, we have previously shown that some types of sarcomas, such as synovial sarcomas, neural tumors, and gastrointestinal stromal tumors, are very homogenous, based on their gene expression profile. In contrast, others, such as liposarcomas, leiomyosarcomas, or malignant fibrous histiocytomas, show considerable heterogeneity and often overlapping molecular features.[16]

The nine cases of DFSP in this study clustered closely together relative to a diverse set of non-DFSP sarcomas based on their gene expression patterns. These tumors were clearly separable from histologically similar STT by the expression of a distinct set of genes, including *PDGFβ*, one of the fusion partners of the *COL1A1:PDGFβ*

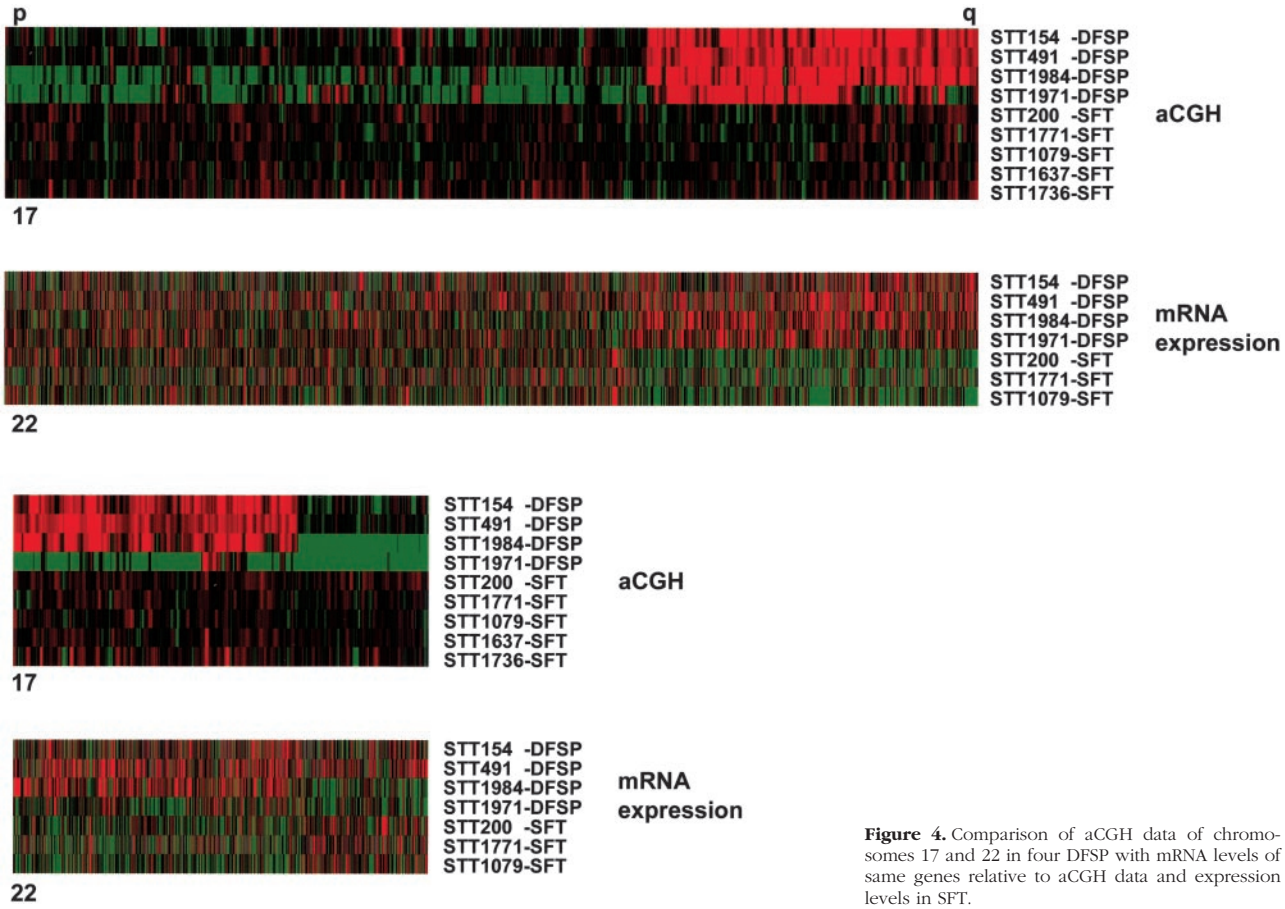


Figure 4. Comparison of aCGH data of chromosomes 17 and 22 in four DFSP with mRNA levels of same genes relative to aCGH data and expression levels in SFT.

translocation. This finding reemphasizes the important role that the fusion product is thought to play in the tumorigenesis of DFSP. In addition, *PDGFBR*, the receptor for PDGFβ, was highly expressed in DFSP, suggesting that an autocrine stimulatory loop may contribute to the tumorigenesis. In a previous study we had made a similar observation for synovial sarcoma, where *SSX*, one of the fusion partners of the t(X;18) *SYT:SSX* translocation, was present in the cluster of relatively highly expressed genes that distinguished synovial sarcoma from the other STT.[16]

To investigate the potential utility of gene expression profiles in the differential diagnosis of DFSP, we also analyzed in this study four tumors that can be misdiagnosed as DFSP. The DNA microarray analysis clearly showed that these tumors were molecularly distinct from DFSP.

cDNA microarray analysis provides an efficient way to search for new diagnostic markers for routine immunohistochemistry. CD34 is a commonly used marker in the diagnosis of DFSP but, like many single markers, it is neither completely sensitive nor specific.[39] The characteristic gene expression pattern of DFSP included several new candidate diagnostic markers, including *APOD*, *EGR2*, *PLA2R1*, and *MEOX1*. Antibodies against APOD stained tumor cells of all DFSPs in this study, but did not stain the fibrous histiocytoma, despite the high levels of APOD mRNA detected in this tumor. A more detailed description of the reactivity of the APOD antibodies will be described elsewhere (West et al, manuscript in preparation).

Several genes that are normally expressed during early embryogenesis and involved in somitogenesis, including *MEOX1*, *HEY1*, and *HEYL*,[40–44] were characteristically expressed in DFSP. During murine embryogenesis, *MEOX1* is expressed in the dermomyotome[40–42] and in sclerotomally derived cells and muscle cells that form the body wall,[41] perhaps paralleling the predominant localization of DFSPs in the dermis of the trunk, and suggesting the possibility that DFSPs originate from these early embryonic mesenchymal cells. In a genome-wide gene expression study of normal human vasculature, *HEY1* and *HEYL* were found to be relatively highly expressed in the microvascular endothelial cells of skin, dermis, lung, and intestine (Jen-Tsan Chi, manuscript in preparation). Preliminary studies have shown that antibodies against HEY1 react with DFSP tumor cells (data not shown).

DFSPs have already been characterized by several cytogenetic studies.[23] The presence of a ring chromosome has been the most consistent genetic abnormality identified in DFSP, being present in more than 75% of cases, sometimes as the only detectable cytogenetic abnormality.[23,45,46] By conventional cytogenetics and comparative genomic hybridization, the ring chromosome was shown to be composed of sequences from

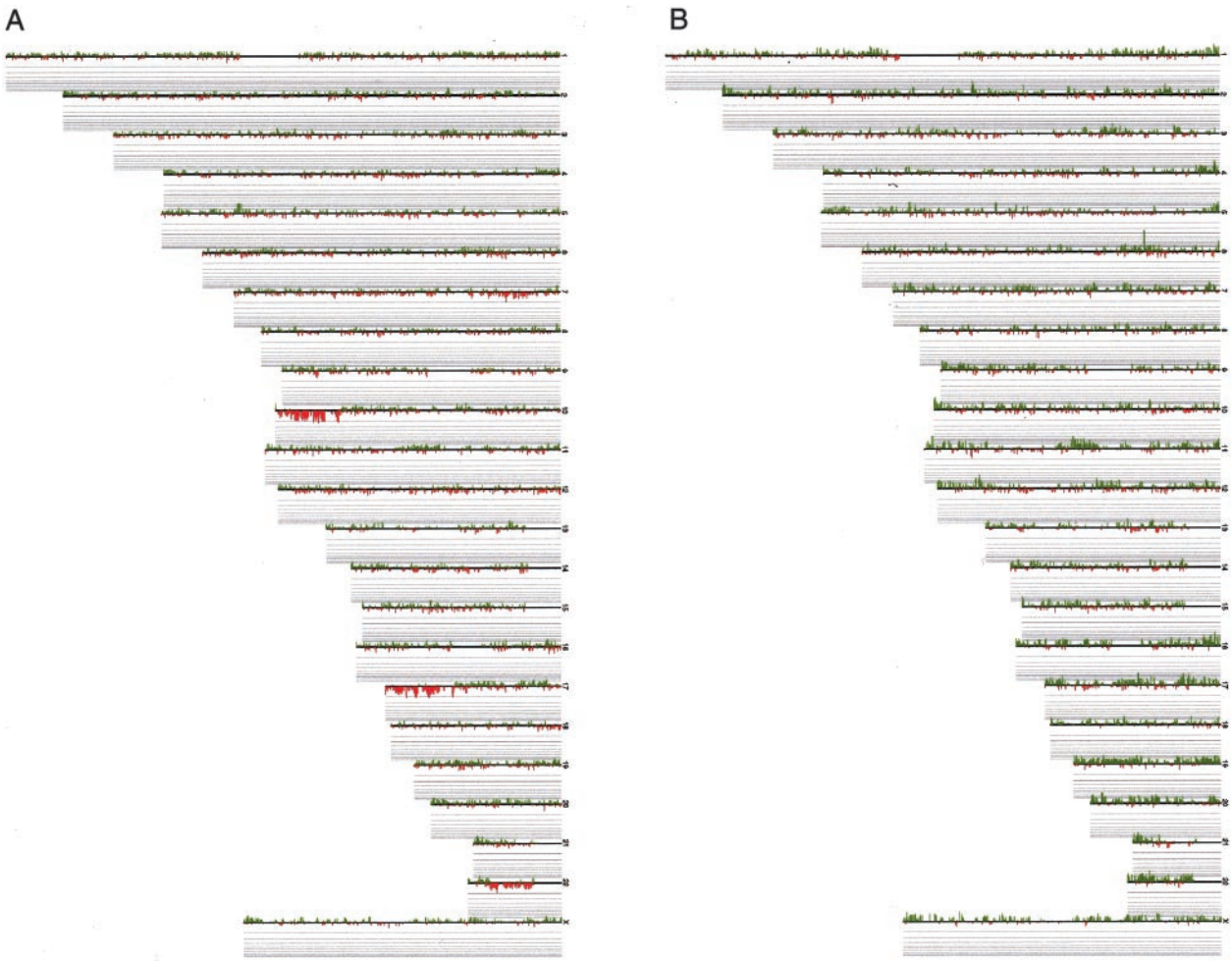


Figure 5. Array-based DNA copy number profiles of DFSP STT154 (**A**) and fibrous histiocytoma 089 (**B**). Individual cDNAs are arranged according to their known location along human chromosomes, to produce a representation of tumor DNA copy number changes mapped onto the normal human genome. Each **vertical line** (red or green) indicates the position of a single cDNA clone, and the length of the line corresponds to the $\log_{10}$ fluorescence ratio of tumor *versus* reference DNA for that locus, reported as a moving average of five adjacent clones. **Red lines** indicate areas of amplification and **green lines** indicate areas of deletion. **A:** The DFSP shows characteristic increase in material on chromosomes 17 and 22. **B:** The fibrous histiocytoma shows absence of significant amplification of chromosomes 17 and 22. Raw data from all paraffin DNA aCGH experiments can be downloaded from SMD, and figures of all aCGH experiments and histology in this paper can be seen at http://genome-www.stanford.edu/DFSP/.

chromosomes 17 and 22,[12,47] leading to the recognition that the fusion of *COL1A1* and *PDGFβ* in DFSP is related to formation of the ring chromosome, or in occasional cases leads to a t(17:22) translocation.[5] Deregulated expression of wild-type PDGFβ protein has transforming activity in NIH3T3 cells[7,8] and is thought to play a critical role in the genesis of DFSP, but it is not clear whether other genetic abnormalities are also required for tumor development. Nishio et al[10] have performed conventional comparative genomic hybridization on 12 cases of DFSP, finding 0 to 3 genetic abnormalities per tumor (mean 1.9). Besides changes in chromosomes 17 and 22, they found gains of copy number of chromosome 8q24.1-qter in three tumors. They did not identify any instances of loss of DNA sequences. By array CGH analysis, we found that abnormalities of chromosomes 17 and 22, consisting of an amplification of large regions of chromosome 17 and 22 bounded by the genes involved in t(17;22), *COL1A1*, and *PDGFβ*, were present in all four cases we studied. This suggests that these abnormalities may have a role in tumorigenesis, in concert with deregulated expression of PDGFβ.

Previous studies have shown that paraffin-embedded tissue can be used for array CGH analysis using 2400 element BAC arrays.[48] We found that formalin-fixed, paraffin-embedded material is also suitable for array CGH using higher resolution genome-wide cDNA arrays, expanding the potential application of array CGH to large-scale retrospective studies of cancer. The average resolution of aCGH can be estimated by dividing the haploid genome size (1.5 billion bp) by the number of mapped genes on the array (~26,000), which equals ~60 kb. This is a conservative estimate of resolution, because genes are generally more closely spaced in the gene-rich portions of chromosomes. By using this conservative estimate, array CGH has a resolution >150 times that of conventional CGH, which is estimated at 10–20 MB for deletions.[49] The aCGH technique can thus be used to distinguish DFSP from lesions in its differential diagnosis,

Table 3. 31 Genes Amplified on chr17 or 22 and Differentially and Highly Expressed in DFSP

Gene symbol	Gene name	Region of amplification
MEOX1	Mesenchyme homeobox 1	17q21
SPAG9	Sperm-associated antigen 9	17q21
ESTs N26608		17q23
ESTs AI339225		17q23
MS12	Musashi homolog 2	17q24
POPX1	Partner of PIX1	17q24
ESTs AA046023		17q24
ESTs N51514	LOC51136 PTD016 protein	17q24
TBX2	T-box 2	17q24
ESTs AI203113		17q24
AXIN2	Axin 2 (conductin, axil)	17q24
PRKCA	Protein kinase C, α	17q25
ESTs R98003		17q25
ABCA9	ATP-binding cassette, sub-family A (ABC1), member 9	17q25
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	17q25
CEP4	Cdc42 effector protein 4; binder of Rho GTPases	17q25
TTYH2	Tweety homolog 2	17q25
FDXR	Ferredoxin reductase	17q25
ESTs AA452113		17q25
FLJ20753	Hypothetical protein FLJ20753	17q25
RAB40B	RAB40B, member RAS oncogene family	17q25
GRB2	Growth factor receptor-bound protein 2	17q25
ESTs AI192496		17q25
PNUTL1	Peanut-like 1	22q11
ARVCF	Armadillo repeat gene deletes in velocardiofacial syndrome	22q11
ESTs AA443624		22q11
ESTs H87363		22q11
BK65A6.2	Sushi domain (SCR repeat) containing	22q11
RBM9	RNA binding motif protein 9	22q12
ESTs AI003064		22q12
PDGFβ	Platelet-derived growth factor β polypeptide	22q13

in cases where only formalin-fixed material is available, as is shown here for five cases of fibrous histiocytoma.

The use of the same cDNA microarrays for array CGH and gene expression profiling allows correlation of changes of gene copy number with gene expression on a genome wide scale. Pollack et al[21] were able to show a strong correlation between copy number changes and gene expression, and concluded that at least 12% of variation in gene expression among the breast carcinomas examined was directly attributable to changes in gene copy number. Unlike breast carcinomas, DFSPs show one consistent genetic abnormality (*COL1A1-PDGFβ* fusion) with relatively few other genetic alterations. Examination of gene expression in DFSP identified elevated expression of *PDGFβ* as a consistent feature. Array CGH showed amplification of the *PDGFβ* gene. In addition, 31 of the 259 genes that we found by SAM analysis to be consistently more highly expressed in DFSP than in other STT were located in the amplified regions of chromosomes 17 and 22. This highlights the potential role of other genes in the amplified regions of chromosomes 17 and 22 in DFSP tumorigenesis, as suggested previously by others.[11] Several of these 31 genes appear particularly worthy of further attention. *TBX2* (Table 3), a gene in the amplified region of chromosome 17, encodes a transcription factor that can immortalize cell by down-regulating *CDKN2a* ($p14^{ARF}$) and that has been found to be expressed at an elevated level in a subset of human breast carcinomas.[50] *TBX2* is highly expressed in DFSP. *Protein kinase C α* is encoded by another of the 31 genes that are both amplified and highly expressed in DFSP. *PRKA* has roles in regulation of cell growth, differentiation, and transformation.[51,52] Although a generic protein kinase C inhibitor (calphostin C) does not inhibit DFSP cell growth *in vitro*,[53] an anti-sense oligonucleotide that specifically inhibits expression of *PRKA* has been shown to be active in several phase I studies in patients with a variety of malignancies.[54,55] *GRB2*, amplified on chromosome 17 (Table 2) and highly expressed in DFSP, belongs to the group of proteins with SH2 and SH3 domains that link tyrosine kinases to intracellular pathways.[56] For instance, after activation of a PDGFRB receptor, GRB2 binds as an adaptor molecule to the receptor and then complexes with SOS1, which leads to RAS activation (see web figure 4).[57] In a mouse model that used the polyomavirus middle T antigen to induce mammary carcinomas, the onset of mammary tumorigenesis was significantly delayed in mice heterozygous for an inactivating Grb2 mutation.[58] The hypothesis that high *GRB2* expression in DFSP contributes to tumorigenesis in DFSP may warrant further investigation. *PRKA* and *GRB2* both act downstream of the PDGF receptor (web figure 4).

A single case of DFSP (STT3053) was noted to lack PDGFβ overexpression. This finding raises the question whether another signaling gene mutation that might substitute for PDGFβ could be present in this case. A similar situation has been described in gastrointestinal stromal tumors where it was recently reported that PDGFRA activation mutations could substitute for the usual KIT mutations found in these tumors.[58]

In summary, DNA microarray analysis of global gene expression patterns and gene copy number changes in DFSP has provided new molecular criteria for the diagnosis of this malignancy, suggested potential clues to its pathogenesis, and identified new candidate molecular targets for therapy.

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