Ph.D Thesis

TRANSFORMED DERMATOFIBROSARCOMA PROTUBERANS: CLINICOPATHOLOGICAL AND MOLECULAR PATHOLOGICAL ANALYSIS

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INTRODUCTION, BACKGROUND

Dermatofibrosarcoma protuberans (DFSP) is a fibrohistiocytic tumour of intermediate malignancy. DFSP is most often diagnosed in individuals between the ages of 20 and 50 years and is seen mainly on the trunk and the proximal extremities. The classic histological features comprise a monotonous storiform growth pattern of cytologically uniform tumour cells with hyperchromatic and elongated nuclei and a characteristic honeycomb pattern of infiltration into subcutaneous fat. Immunhistochemically, DFSP is characterized by positive reaction for vimentin and CD34.

DFSP is characterised clinically by locally aggressive growth and a high rate of local recurrence, but distant metastases (1-4%) and tumour related deaths are very rare. Recurrence is common in patients with DFSP and the literature suggests an incidence of 20-50%. There seems to be a poor correlation between the size of the tumour and the recurrence rate but the completeness of excision and the distance of margins from the tumour have been reported to affect it. Several reports suggest that excision with at least 2 cm of free margin (so-called wide local excision) reduces the relapse rate significantly.

DFSP is morphologically heterogeneous; several rare variants have been described that are important to recognise to avoid misdiagnosis with more aggressive tumours. Most of the variants of DFSP are not associated with significant differences in clinical behaviour.

A small number of cases of DFSP contain areas of fibrosarcoma (FS) or, more rarely, malignant fibrous histiocytoma (MFH). During the past two decades, several case reports and small series of DFSP with FS-like or MFH-like areas have been published. These variants of DFSP have undetermined prognostic relevance.

In 1951, Penner reported a case of metastasising DFSP containing areas that were histologically indistinguishable from fibrosarcoma. More recently, Ding et al. suggested a higher recurrence rate in cases of FS transformation of DFSP, but the surgical treatments were not well described in their report. Similar observations were documented by Mentzel et al. and Pizarro et al., but in their studies either only a minority of cases received wide local excision, which is the standard of practice of treatment of DFSP, or treatment details were not provided.

In contrast, Connely and Trotter reported that recurrences were dependent on the adequacy of resection. One of the largest studies by Goldblum et al. found no prognostic differences between conventional DFSP and DFSP containing sarcoma treated by wide local excision.

Recent cytogenetic studies have revealed that reciprocal translocation, t(17;22)(q22;q13), and a supernumerary ring chromosome derived from the translocation, r(17;22) are highly characteristic of DFSP. The chromosomal re-arrangements fuse the collagen type Iα1 (COL1A1) and the platelet-derived growth factor B-chain (PDGFB) genes.

The fusion causes deregulation of the PDGF gene by removing upstream sequences from exon 2 and placing it under the direct control of

the COL1A1 gene. This gene rearrangement results in uncontrolled production of the growth factor, which seems to be the most important genetic event in the pathogenesis of DFSP.

Detection of the COL1A1-PDGFB chimeric mRNA by a reverse transcription-polymerase chain reaction (RT-PCR) assay has also been proved to be a useful diagnostic marker for DFSP.

Moreover, the COL1A1-PDGFB fusion transcript has been demonstrated not only in conventional DFSP, but also in a small series of DFSP-FS using reverse transcription based conventional polymerase chain reaction. Nothing is known about the status of COL1A1-PDGFB chimeric gene in the malignant fibrous histiocytomatous areas of DFSP-MFH.

On the other hand, real time PCR plays an increasingly important role in clinical testing, because it is objective, rapid, cost effective and can be performed on small tissue samples. Advantages of real time methods include the elimination of the need for post-PCR processing and confirmation of PCR products. Because real time PCR is sensitive and uses small fragments of cDNA for amplification, it is also well suited for use with formalin-fixed, paraffin wax embedded material.

AIMS

1. To investigate the biological behaviour of transformed DFSP by analysing a series of patients with longterm follow up.
2. To analyse the effect of local treatment on the recurrence rate of transformed DFSP.

3. To conduct a real time polymerase chain reaction (RT-PCR) assay for the COL1A1-PDGFB fusion transcript in a series of DFSP containing sarcoma.

4. To determine whether the chimeric gene could be identified in both components of DFSP-FS and DFSP-MFH.

MATERIALS AND METHODS

Patients

Eighty seven consecutive cases of DFSP were retrieved from the routine histological files of the Department of Pathology, Medical and Health Science Center, University of Debrecen. Formalin-fixed, paraffin wax embedded material was available for evaluation. Paraffin wax embedded sections from all cases were processed routinely and stained with haematoxylin-eosin. The haematoxylin and eosin stained slides of each specimen were reviewed to identify sarcomatous changes. The following diagnostic criteria were used: (1) the presence of classical morphological features of DFSP and (2) the presence of sarcomatous change in at least 5% of the lesion. FS change was defined as mitotically active cellular area composed of spindle cells arranged in fascicular pattern. MFH transformation was defined as mitotically active cellular area composed of pleomorphic, undifferentiated cells. Eight cases that fulfilled these criteria were included in this study.

In these eight cases the following features were recorded: tumour size; area of the sarcomatous transformation; estimated percentage of DFSP

and sarcoma; the depth of infiltration; mitotic figures (MF)/10 high power fields (HPF), counted in randomly selected areas of both components. In addition, we graded all sarcomas using Trojani grading system. This grading system uses the parameters of tumour differentiation, necrosis and mitotic activity.

Immunohistochemical analysis was also performed. The dewaxed sections were incubated at room temperature for 30 minutes with the following primary antibodies: vimentin (clone V6, Dako, diluted 1:40), α-smooth muscle actin (clone αsm1, Novocastra, diluted 1:50), desmin (clone D33, Dako, diluted 1:40), CD34 (clone QBEnd/10, Novocastra, diluted 1:25), factor XIIIa (FXIIIa, polyclonal, Calbiochem, diluted 1:200). We used high-pressure antigen retrieval with citrate buffer (pH 6.0) and then a standard peroxidase labelled streptavidin-biotin (ABC) method with VIP (SK 4600, Vector Laboratories) chromogene. The ratio of CD34 and FXIIIa immunoreactive cells to the tumour cell population was graded semiquantitatively on a scale from 0 to 3+.

Clinical details and follow-up data were obtained from hospital records, referring pathologists and clinicians.

Control group

We selected 20 cases of conventional DFSP from the remaining 79 cases, the criterion for selection being treatment by wide local excision. All tumours consisted of conventional DFSP without sarcomatous change. We

recorded the clinical details of the patients and the maximum diameter of the tumors.

Microdissection

Microdissection was performed on sections in the same way as previously described by Wang et al. Briefly, using RNA-free conditions formalin-fixed, paraffin wax embedded samples were cut in 10 μm thick sections on a microtome with disposable blade. Sections were collected on clean and sterile treated glass. The samples were dewaxed in two changes of xylene for 10 minutes, rehydrated in 100% ethanol, 90% ethanol, and 70% ethanol for 5 minutes each, stained lightly with haematoxylin and eosin (H&E) for identification, rinsed in RNase free water for 30 seconds, and finally immersed in 100% ethanol for 1 minute. The sarcomatous areas were cut out from the sections using sterile fine needles.

Five cases of conventional DFSP were included as a control group for molecular analysis.

Extraction of total RNA

Dewaxed, microdissected sections were placed in a sterile tube, and resuspended in a proteinase K-containing buffer, and incubated at 60 °C for 16 hours until the tissue was completely solubized. RNA was purified using the EZ1 Biorobot with EZ1 RNA Tissue Mini Kit (Qiagen, Hilden, Germany).

Probe and primer design

Primer and probe sequences were chosen with the software Primer Express (Applied Biosystems, Foster City, CA, USA) based on the length between the exons within the region encoding the α-helical domain (exon 5 through exon 49). In order to eliminate the need for multiple probe sequences we used one probe sequence complementary to the 5` end of PDGFB gene. Primers and probes were purchased from IDT (Coralville, IA, USA).

One step real time PCR

PCR amplification was performed in duplicate using a 96-well plate (Applied Biosystems, Foster City, CA, USA) with a 50 μl final reaction mixture containing 10 μl extracted RNA, 25 μl 2x Quantitect Probe PRT-PCR Mix, 0.5 μl Quantitect RT Mix, 0.7-0.7 μl (1μM-1μM) from forward and reverse primers and 0.3 μl (0.2μM) probe and additionally 17.8 μl RNA-free water (Quantitect Probe RT PCR Kit, Qiagen, Hilden, Germany). The integrity of RNA was evaluated by running a parallel PCR for the ubiquitously expressed 36B4 gene.

The reaction started with reverse transcription for 30 min at 50 °C and followed by denaturation at 95 °C for 15 min. After the initial step, 40 cycles of amplification were performed with extension for 30 s at 76 °C, denaturation at 94 °C for 15 s, and annealing/detection at 56 °C for 30 s. The data were analyzed by Sequence Detection Software (Applied Biosystems, Foster City, CA, USA).

RESULTS

Clinical features of transformed DFSP

We examined five patients of DFSP-FS and three with DFSP-MFH. There were six women and two males and their age at diagnosis ranged from 26-62 (median, 41). Most patients had a history of superficial, slowly growing, painless mass; unusual clinical signs were not detected. The location of the lesion included the trunk (six cases) and the lower extremity (two cases). Tumour size ranged from 3.5 to 8 cm (median, 4). After diagnosis, six of the patients were treated by wide local excision and two by incomplete local excision and radiotherapy with or without chemotherapy. Surgical margins were positive in the last two cases.

Follow up ranged from four to 36 years after the first operation, and in seven of eight cases it was more than five years.

Recurrences occurred in three of eight cases: in two patients with positive surgical margins and in one patient who was treated by wide local excision. The overall recurrence rate in sarcomatous variants was 37.5%, but this was only 17% in patients who were treated by wide local excision.

The interval to recurrence ranged from nine months to four years. One patient developed three recurrences, two recurrences occurred in one case and one recurrence only was seen in one patient.

There were recurrences in two of the three DFSP-MFHs (recurrence rate of 66%) and the surgical margins were positive in recurrent cases.

Recurrence was seen in one of the five patients with DFSP-FS (recurrence rate of 17%). The patient was treated by wide local excision with negative margins.

Control group for clinicopathological analysis

We examined 20 cases of conventional DFSP. The patients comprised 13 women and seven men and their ages ranged from 23-48 years (median, 35). The lesions were located on the trunk (10 cases), the lower extremity (three cases), the head and neck region (three cases), the shoulder region (two cases), the inguinal region (one case), and the upper extremity (one case). Most patients had a history of superficial, slowly growing, painless mass. Tumour sizes ranged from 2 to 5 cm (median, 3.585). After the diagnosis, all patients were treated by wide local excision. Surgical margins were negative in all cases.

There were recurrences in three of 20 cases (recurrence rate of 15%). Recurrence occurred between 14 and 18 months after primary surgical intervention (mean, 19).

The follow-up interval ranged between 3 and 14 years (mean, 8.6).

Histopathology

The soft tissue tumour diagnoses were made according to the description of Weiss and Goldblum.

All tumours consisted of areas of ordinary DFSP composed of uniform spindle-shaped tumour cells with slender nuclei arranged in a

storiform growth pattern, with intercellular collagen deposition and small capillary blood vessels scattered throughout. Characteristic honeycomb or lace-like infiltration into underlying subcutaneous fat was seen at least focally. There was little nuclear pleiomorphism with low mitotic activity. Mitotic counts ranged from 1 to 3 MF/10 HPF (median, 1.94). Myxoid change was conspicuous in one case. The ratio of DFSP in the complex lesions varied from case to case from 45% to 75% (median, 54%).

Sarcomatous change was found de novo in all cases.

The FS area of the tumour showed a fascicular (no longer storiform), and highly cellular histological pattern, frequently exhibiting a characteristic herringbone pattern with the fascicles crossing at acute angles. Tumour cells in FS areas had slightly more plump nuclei than those of tumour cells in areas of ordinary DFSP. FS area showed moderate polymorphism and increased mitotic activity. Bizarre cellular features were not seen in FS. The FS areas were found in the subcutaneous part of the tumour, and the dermis was uninvolved in all cases. The interface between the DFSP and FS was sharp in three cases, and indistinct in two. Focal myxoid change of the FS areas was found in two of four cases.

In the MFH areas of the tumour the cells were large and pleomorphic. In these areas, multinucleated giant cells were common, and the mitotic activity was increased. MFH areas were found in the subcutaneous part of the tumour, the dermis was involved in two cases. The border between the DFSP and MFH was indistinct in two cases, and sharp in one case. Focal myxoid change was found in two cases of MFH.

In general, proliferative activity generally was higher in sarcomatous areas. In areas of classic DFSP the mitotic count ranged from 1 to 3 mitoses/10 HPF (mean, 1.875) whereas in FS and MFH areas the mitotic count ranged from 9 to 16 MF/10HPF (median, 12). In cases showing high proliferative activity in sarcomatous areas neither local recurrence, nor metastasis occurred.

Frank tumor necrosis and areas of extensive hemorrhages was seen in one case.

The metastatic tumor from one patient was composed exclusively of MFH without DFSP. The histopathological features of metastatic tumour were identical to the original MFH described above.

The grade of sarcomatous area was 1 in one case and 2 in 7 cases.

Immunohistochemistry

Vimentin was positive in conventional DFSP and in the FS or MFH areas of all of cases. Tumour cells in conventional DFSP areas stained strongly for CD34 in all cases, whereas in FS areas only three cases were positive. There was no CD34 positivity in MFH areas. Factor XIIIa reactivity was seen only in scattered spindle cells in conventional DFSP areas of all cases, whereas we found a diffuse strong reaction in more than 2% of cells in MFH areas, but not in FS areas. FXIIIa positive cells belong to the stromal component of the tumours as shown in our pervious work. All tumors were negative for desmin and α smooth muscle actin.

Molecular pathological findings

All cases except one contained amplifiable RNA as determined by successful amplification of 36B4 gene.

PCR revealed that transformed DFSPs show translocations between exons 27, 32, 34, 40, 47 of COLIa1 gene and exon 2 of PDGFB gene in the sarcomatous area of DFSP.

The cases from the molecular pathology control group showed translocations between exons 29, 32, 34, 38, 42 of COL1A1 gene and exon 2 of PDGFB gene.

DISCUSSION

In our study, we evaluated the clinicopathological profile of conventional DFSP, and DFSP containing sarcoma (FS or MFH) with an emphasis on the longterm follow up. Sarcomatous change occurred in eight of the 87 DFSP cases in our study. We analysed eight patients with sarcomas arising in DFSP (five cases of DFSP-FS and three cases of DFSP-MFH) and 20 patients with conventional DFSP. There were no remarkable differences in age and anatomic site between DFSP containing sarcoma and those with ordinary DFSP. The sarcomatous change presented in de novo cases, and not as recurrences. All conventional DFSPs were treated by wide local excision, and the recurrence rate was found to be 15%, which is lower than the published rate.

For the clinicopathological correlations, we chose to study six patients of DFSP containing sarcoma, who were treated by wide local

excision and two other patients who were treated by incomplete local excision(s) and additional radiotherapy with or without chemotherapy. In all of our transformed cases the sarcomatous area constituted a significant portion of the tumor and ranged from 25% to 70%. The length of our follow up period was sufficient enough to encompass the initial three year period, during which the most recurrences occur.

In our study, there were recurrences in three cases of transformed DFSP, and the total recurrence rate of DFSP-FS and DFSP-MFH was 37.5%. The total recurrence rate of DFSP containing sarcoma in our study was significantly higher than that of our control group. Unfortunately, only one case of recurrent DFSP containing sarcoma was treated with wide local excision. When the treatment was simple local excision, the recurrence rate was 100%. Five patients were free of local recurrences and/or metastases. The metastasis seen in one patient with DFSP-MFH could be attributed to the aggressive nature of the transformed tumour. The overall recurrence rate of DFSP containing sarcoma was 17% when treated by wide local excision. This observation indicates that DFSPs with sarcoma that lack positive surgical margins may have the same clinical behaviour in longterm follow up as conventional DFSP treated by wide local excision, but DFSP with sarcomatous foci is a more aggressive neoplasm, which had a 12.5% metastatic rate in our study. However, the metastatic rate in conventional DFSP is less than 3%.

Immunohistochemical demonstration of CD34 is an important feature diagnosing DFSP. In all our cases of conventional DFSP areas of

strong CD34 positivity in the majority of tumour cells was noted, whereas the sarcomatous areas showed scarce CD34 reactivity in three of eight cases. This can be explained by loss of expression of CD34 during tumour dedifferentiation.

Recurrence and metastatic potential were not influenced by the depth of invasion, the amount of sarcomatous area, or the histological grade of the sarcomatous component.

Local treatment is a crucial determinant of the behaviour of tumors in general, and in the case of low or high grade extremity sarcomas. The recurrence potential of DFSP is directly related to extent of resection. Micrographic surgical resection could theoretically reduce the risk of the recurrence to zero, although it can be extremely difficult to determine a positive margin considering the fact DFSP has fibroblast-like morphology.

In the absence of recurrence, the risk of metastasis is greatly reduced, since metastasis never occurs without antecedent local recurrence. Thus, in our opinion wide local excision with negative margins reduces, or sometimes, eradicates the risk of local recurrence or dissemination.

However, the tumours that are removed by wide local excision with clear margins probably represent less aggressive subset of lesions, as shown in high grade extremity sarcomas.

Recent cytogenetic and molecular studies have revealed that fusion of COL1A1 gene with PDGFB gene is very characteristic of DFSP. COL1A1 gene encodes the major component of type I collagen, and PDGFB is a

potent mitogen for a number of cell types. The location of breakpoints within COL1A1 varies greatly, but is always limited to the region encoding the alpha-helical domain. The exons of this COL1A1 gene segment end at the last base of the codon. The PDGFB segment of chimeric transcript always starts with exon 2. The resulting COL1A1-PDGFB fusion is in-frame, because exon 2 of PDGFB starts at the first base of codon 22. The COL1A1 part of the fusion gene serves to act as an active promoter for PDGFB. The translocation removes negative regulatory segments in the 5' end of the PDGFB gene that enhances protein production. Wang et al. demonstrated that the COL1A1-PDGFB transcripts are preserved in the FS areas of most DFSP-FS using conventional PCR reactions and they suggested that COL1A1-PDGFB chimeric gene is still involved in the fibrosarcomatous transformation of DFSP and the deregulated PDGFB continues to act as a growth factor in the process of tumor progression.

Although the COL1A1-PDGFB fusion transcript has been studied in DFSP, giant cell fibroblastoma (juvenile form of DFSP), DFSP containing FS, and superficial fibrosarcoma, it has not been investigated in the DFSP-MFH variant.

In this study, we have demonstrated that the COL1A1-PDGFB fusion transcripts were detectable not only in ordinary DFSP component of transformed DFSPs. We found DFSP specific translocation not only in fibrosarcomatous areas, but in MFH areas. The exon 32, exon 34 and exon 40 of COLIA1 gene were found to be involved in DFSP-MFH. This finding

indicates that the COL1A1-PDGFB fusion gene can be involved in the pathogenesis of high-grade transformation of DFSP.

We also investigated five cases of DFSP containing FS. We found no amplifiable cDNA in one case (case 1) probably due to the age of the specimen. 4 cases showed the characteristic translocation between various exons (exon 27, 32, 40, 47) of COLIA1 gene and PDGFB gene.

Analysing seven cases with transcriptable RNA, we could not demonstrate specific, sarcomatous change related translocation, but from a diagnostic standpoint, this assay can be particularly useful in confirming the diagnosis of sarcomatous DFSP.

On the other hand, previous reports described a basic PCR reaction with reverse transcription and sequence analysis, which make a multistep way of detection of the specific translocation. In theory, real time PCR combines the objectivity of fluorescence detection with the simplicity of original PCR reaction, and results obtained using fluorescence based PCR reagents are accepted as the standard of detection of genetic alterations. Real time PCR makes post-PCR manipulations of the PCR products unnecessary, which reduces the analysis time and lowers the risk of PCR contamination.

The sensitivity of conventional PCR and real time PCR differs. Real time PCR uses smaller amount of transcriptable RNA than basic PCR reactions. It is important, because RNA isolated from paraffin-embedded tissue blocks is of poor quality because extensive degradation of RNA can occur before completion of the formalin fixation process. Moreover

formalin fixation causes cross-linkage between nucleic acids and proteins, making subsequent RNA extraction, reverse transcription problematic. In addition, real time PCR can be useful in quantitation of gene expression, but in formalin-fixed, paraffin embedded tissues it has serious limitation due to the degradation of RNA.

In summary, we have shown that sarcomatous change in DFSP represents a form of tumour progression, and is associated with a worse prognosis than ordinary DFSP. The degree of aggression of DFSP containing sarcomas may be related to the histological grade of sarcomatous transformation. Even though transformed DFSP is a more aggressive tumour, the prognosis can be influenced by the extent of excision, so that there may be little increased risk for relapse and dissemination over that of conventional DFSP. In addition, we have shown that real time PCR based gene analysis may be utilized in the diagnosis of DFSP and transformed DFSP cases. We have demonstrated the COL1A1-PDGFB fusion transcript in DFSP containing FS or MFH which supports the theory of the common clonal origin of the two components.

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