Characterization of Molecular Abnormalities in Human Fibroblastic Neoplasms: A Model for Genotype-Phenotype Association in Soft Tissue Tumors


INTRODUCTION

The spectrum of the fibroblastic family of neoplasms includes desmoid tumors and LG-2 and HG-FS, each of which show distinctive differences in biological behavior. Desmoid tumors are clonal neoplasms (1) with a low cellularity and rare mitoses that are locally aggressive but lack a metastatic potential. The significance of surgical resection with positive resection margins remains controversial, and it is still uncertain whether or not this is a predictor for local recurrence of these tumors (2–5). FS are generally more cellular and mitotically active and may display nuclear polymorphism. They invade locally and have the potential to metastasize. Positive margins of resection are negative predictors of patient survival in these tumors (6, 7). LG-FS are less aggressive and less prone to metastasize than HG-FS (6), and LG-FS are sometimes misdiagnosed as desmoid tumors, especially in small nonrepresentative biopsies.

Desmoid tumors and FS are different tumor entities. The biological determinants of aggressive behavior may be attributable to different genetic alterations of regulatory molecules of proliferation, cell-cycle control, and apoptosis. Recent cell culture studies have demonstrated the significance of certain molecular elements for converting a nonmalignant fibroblastic cell into a cancer cell in vitro (8). From a more clinical standpoint, it is relevant to identify molecular changes accounting for the phenotypic characteristics of human tumors in vivo.

In the present study, we analyze molecular markers associated with proliferation, cell cycle control, and apoptotic programs. Ki-67 is an indicator of cellular proliferation and has been shown to be associated with prognosis in patients with HG sarcomas (9, 10). The tumor suppressor genes p53 and pRB are negative regulators of the cell cycle involved in controlling the transition from G0/G1 to S phase. Loss of function of these genes based on mutations, chromosomal deletions, or inactivation through other molecules is very common in human cancers, particularly in sarcomas, and can be associated with patient prognosis (9, 11–13). The antiapoptotic protein BCL-2 is part of a balanced system of apoptotic regulators and is linked to the p53 pathway. BCL-2 down-regulation leads to induction of apoptosis, whereas its overexpression prevents cells from entering the apoptotic pathway, a phenomenon frequently found in human cancers (14, 15).

The final objective of our study was to identify potential molecules that may be relevant for the phenotypic and biological behavior and distinction of these fibroblastic tumors and to define their prognostic significance. The immunoreactivity patterns of these markers have been correlated with clinical data and patient outcome.

ABSTRACT

Desmoid tumors and fibrosarcomas (FS) are part of a wide spectrum of disordered fibroblastic growth that display striking clinical and phenotypic differences. This study was designed to characterize molecular abnormalities that are associated with these differences and to determine their clinical relevance. A cohort of 24 desmoid tumors and 25 low-grade (LG) and 14 high-grade (HG) FS that were clinically and pathologically well characterized was analyzed for alterations in expression of Ki-67, Bcl-2, retinoblastoma gene product (pRB), and p53 by immunohistochemistry. LG-FS and HG-FS showed abnormal expression of Ki-67 (32 versus 86%), Bcl-2 (48 versus 57%), and pRB (56 versus 93%). In contrast, desmoid tumors showed a normal phenotype with these markers. p53 overexpression was identified in 20% of LG-FS and in 29% of HG-FS cases but only in 4% of desmoid tumors. There was an increasing trend in the proportion of abnormal expression of Ki-67, Bcl-2, pRB, and p53 with the increase of tumor aggressiveness from desmoid tumors to LG-FS to HG-FS. The molecular differences between tumor entities were highly statistically significant (P < 0.01). Significant associations between abnormal expression of pRB and recurrence-free survival of LG-FS patients (P = 0.05) and between Ki-67 overexpression and recurrence-free survival for tumors of > 5 cm were observed (P = 0.02). The demonstrated differences of molecular alterations in HG-FS, LG-FS, and desmoids appear to be related to biological aggressiveness of such tumors, and they might be useful to differentiate between histologically similar cases of desmoid tumors and LG-FS. pRB and Ki-67 status may be useful to predict recurrence in certain subsets of patients.

MATERIALS AND METHODS

Clinical and Pathological Data. The cohort that was analyzed consisted of well-characterized fibroblastic neoplasms that included desmoid tumors (n = 24), a group of LG-FS (n = 25), and a group of HG-FS (n = 14) among 63 patients treated at MSKCC between August 1982 and January 1999. Thirty-nine patients were treated with surgery alone, 24 received additional radiation therapy and 4 of these 24 patients also received chemotherapy. FS groups consisted of the following subtypes: LG-FS included 8 myxofibrosarcomas, 9 conventional FS, 6 fibromyxoid FS (Evans tumor), and 2 sclerosing epithelioid FS, whereas HG-FS included 11 conventional FS, 2 myxofibrosarcomas, and 1 pleomorphic FS. Samples were analyzed from 44 primary tumors, 17 local recurrences, and 2 metastases. From 56 patients, specimens have been resected before adjuvant therapy, and, therefore, adjuvant therapy had no effect on these results. For the remaining seven patients, radiation or chemotherapy was given before resection of disease. Five were treated with radiation after primary resection, and tumor specimens were taken from a recurrence developed thereafter. This was mainly attributable to the referral of patients to MSKCC after primary treatment. The remaining two patients had either neoadjuvant chemotherapy before primary resection or prior radiation for a different disease in the field in which the sarcoma developed. Analysis was done on frozen and on paraffin-embedded tissue. Median age of the cohort was 44 years (range, 10–86 years). The clinical history was reviewed from patient charts to address biology of disease-specific questions. Tissue sections of each specimen were stained with H&E and examined microscopically by two pathologists (C. R. A., J. M. W.). All of the cases were reviewed to evaluate histopathological diagnosis, tumor grade, and quality of the tissue.

Recurrence-free and overall survival were defined as time from primary tumor resection to first recurrence (either local or distant) or death from disease, respectively. Because there was only one death from disease in the LG-FS group and no death of disease in the desmoid group, a comparison

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2 The abbreviations used are: LG, low-grade; HG, high-grade; FS, fibrosarcoma(s); pRB, retinoblastoma gene product; MSKCC, Memorial Sloan-Kettering Cancer Center; RFS, recurrence-free survival; IHC, immunohistochemistry.
between categories in terms of overall survival was not feasible. Therefore, only analyses of RFS were carried out.

**Tissue and IHC.** Tumor samples were obtained fresh following surgical resection, embedded in a cryopreservative solution (OCT Compound; Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C. Five-μm frozen sections were fixed in formalin and used for immunohistochemical analysis (16). In parallel, normal and tumor tissues were embedded in paraffin, and 5-μm sections were also used for immunohistochemical analysis. Tissues and cell lines known to express the antigens under study were used as positive controls. Sections of normal connective tissue were prepared and served as baseline controls. All of the connective tissue samples were negative for the four markers.

For frozen tissue analysis, sections were incubated with 10% normal horse serum for 30 min, and then primary antibodies were applied for 1–2 h. Mouse antihuman monoclonal antibodies to p53 (Ab-2, clone 1801; Calbiochem, Cambridge, MA), pRB (clone 3c8; QED Bioscience, San Diego, CA), underphosphorylated pRB (G99-549; PharMingen, San Diego, CA), Bcl-2 (clone 124; Dako, Glostrup, Denmark), and Ki-67 (Mib-1; Immunotech, Marseille, France) were used. The anti-p53 antibody detects wild-type and mutated p53. The anti-pRB antibody 3c8 detects normal and hyperphosphorylated pRB products, whereas the anti-pRB antibody G99-549 detects only underphosphorylated pRB. Antibodies for Bcl-2 and Ki-67 recognize epitopes from human recombinant peptides of the two proteins. Samples were then incubated with biotinylated antimouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin peroxidase complexes (1:25; Vector Laboratories, Inc.) for 30 min. Diaminobenzidine was used as the chromogen, and hematoxylin as the nuclear counterstain.

For paraffin tissue analysis, sections were deparaffinized, treated with 1% H2O2, immersed in boiling 10% citrate buffer, and incubated in 10% normal horse serum overnight at 4°C. Identical primary antibodies were used as described above, and samples were then incubated with biotinylated antimouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin peroxidase complexes (1:25; Vector Laboratories, Inc.) for 30 min. Again, diaminobenzidine was used as the chromogen, and hematoxylin as the nuclear counterstain. The processing for Ki-67 included trypsinization for 5 min prior to IHC.

Immunoreactivities were classified as continuum data (undetectable levels or 0% to homogeneous staining or 100%) for all of the four markers. Slides were reviewed by several of the investigators (A. H., C. R. A., C. C-C.) and results were scored by estimating the percentage of tumor cells showing characteristic staining. The cutoff values used in this study have been shown to be highly sensitive (9, 17) and were defined as follows: (a) high proliferative Ki-67 index if >20% tumor nuclei stained; (b) p53 nuclear overexpression if >10% tumor nuclei stained; and (c) Bcl-2 cytoplasmic overexpression if >10% tumor cells stained. For pRB, no cutoff value was defined.

Tumors were then grouped into two categories for each tumor entity defined as follows: (a) normal expression (neoplasms below defined cutoff value of immunoreactivity in tumor cells); and (b) abnormal expression (neoplasms above defined cutoff values of immunoreactivity in tumor cells).

**Statistical Analysis.** Associations between clinicopathological parameters and laboratory data were studied using Fisher’s exact test and the Cochran-Armitage trend test (18). Survival analysis was performed by the method of Kaplan-Meier (19), and statistical significance (P ≤ 0.05) was evaluated by log-rank testing for univariate analysis (20).

**RESULTS**

**Clinical and Pathological Analyses.** Median age of the cohort was 44 years (range, 10–86 years). Samples were analyzed from 44 primary tumors, 17 local recurrences, and 2 metastases. Seven of 63 patients received radiation or radiation plus chemotherapy before resection of specimens used for this analysis. No specific patterns of expression of the analyzed molecules were observed in these cases that could be attributable to this treatment. For example, desmoid tumors showed uniformly negative results for all of the markers independent of radiation or chemotherapy.

Thirty-nine tumors were extremity lesions, 17 were located in the trunk, 5 in the head and neck area, and 2 intra-abdominally. Fifty tumors were deep lesions, i.e., below the level of muscular fascia and 8 were superficial; for 5 lesions, the depth was undetermined. The size distribution of the primary tumors of each patient was as follows: <5 cm (n = 16, 25%), ≥5 cm but < 10 cm (n = 20, 32%), ≥10 cm (n = 21, 33%), and for 6 patients, who presented with local recurrences or metastases, the size of the primary tumor was undetermined.

Postresection margins were grossly negative in 51 and grossly positive in 4 patients, whereas in 8 patients, margin status information was not available. Microscopic margins were negative in 34 and positive in 25 patients, whereas for 4 patients, no microscopic margin status was available because of treatment received before the patients were referred to MSKCC.

Median follow-up for the entire group at the time of correlating laboratory results and clinicopathological data was 38 months. Median follow-up for desmoid tumors, LG-FS, and HG-FS was 30 (8–107 months), 43 (1–228 months), and 48 (4–122 months), respectively. None of the desmoid tumor patients, one LG-FS patient, and 8 of the HG-FS patients died of the disease. Of 24 patients with desmoid tumors, 13 developed local recurrence, and there were no metastases. Seven of 25 LG-FS patients developed recurrence of whom 4 had metastases, whereas 9 of 14 HG-FS patients developed recurrence of whom 6 had metastases.

Because there was only one death of disease in the LG-FS group and no death of disease in the group of desmoid tumors, a comparison between categories in terms of overall survival was not feasible. Therefore, only analyses of RFS were carried out.

At last follow-up after completion of treatment at MSKCC from the entire cohort, 45 patients had no evidence of disease (NED), 8 were alive with disease (AWD), 9 died of their disease (DOD), and 1 died of other causes (DOO).

Overall survival for patients with desmoid tumors (n = 24) was 100%. Median RFS for desmoid tumor patients was 26 months with a range of 5–68 months. Only one patient with LG-FS (n = 25) died of the disease. In this group, the median RFS was 156 months. Patients with HG-FS (n = 14) had a median overall survival of 67 months and a median RFS of 10 months.

**Analysis of Ki-67 Proliferative Index.** High Ki-67 proliferative index was found in 20 (51%) of 39 FS but was not detected (0%) in 24 desmoid tumors. High proliferative index occurred more frequently in HG- compared with LG-FS: 8 (32%) of 25 LG lesions displayed >20% nuclear staining for Ki-67, whereas 12 (86%) of 14 HG tumors showed this staining pattern. These data demonstrate low proliferative activity in nonmalignant desmoid tumors compared with increasing activity with gain of tumor aggressiveness in LG and HG malignant FS. Observed increase in proliferative activity from desmoids to HG-FS was statistically significant with a P of <0.01 (Table 1). Pairwise comparison between desmoids and LG-FS and desmoids and HG-FS also showed statistical significance (P < 0.01).

**Analysis of p53 Expression.** p53 protein half-life is short, and expression levels are low in normal cells, and, therefore, IHC cannot detect these normal p53 levels. In cancer cells, most p53 mutations lead to products that are not ubiquitinated, accumulate in the nuclei, and can be demonstrated by IHC. The concordance between p53 mutations detected by IHC and DNA sequence analysis demonstrates the reliability of this assay (21). From the 39 FS analyzed, 9 (23%) cases showed nuclear staining, whereas only 1 (4%) of 24 desmoid tumors was found positive. Stratified for grade, there were 5 (20%) of 25 LG- and 4 (29%) of 14 HG-FS positive for p53. These data show increased frequency of p53 mutations with increase of aggressive biological behavior of tumors, which indicates the relevance of the frequency of molecular changes with increasing tumor aggressiveness. These differences were statistically significant between all of the three groups (P < 0.01; Table 1). Pairwise comparison of groups did not show statistical significance.
Analysis of pRB Expression. Genetic alterations of RB include deletions and point mutations. Patterns of expression of pRB have been classified as wild-type when low nuclear staining is observed, and abnormal when undetectable (because of genetic deletion/mutation) or when producing high nuclear staining (mainly attributable to nonactive, hyperphosphorylated proteins; Refs. 22, 23). In our cohort, 27 (69%) of 39 FS were found to be altered, which showed either lack of pRB or accumulation of hyperphosphorylated pRB. In contrast, none (0%) of the 24 desmoid tumors was found to have abnormal patterns of expression. To demonstrate that the pRB antibody (3c8) detects hyperphosphorylated pRB, all of the highly positive cases were additionally analyzed with an antibody that only detects underphosphorylated (wild-type) pRB (G99-549). These cases were either weakly positive or negative for wild-type pRB, which indicated the dominance of hyperphosphorylated pRB in these cases. The frequency of pRB abnormalities was higher in HG-13 (93%) of 14] compared with LG-[14 (56%) of 25] FS. These differences between the three tumor entities were statistically significant ($P < 0.01$). Pairwise comparison between desmoid tumors and LG-FS ($P < 0.01$) and between LG-FS and HG-FS ($P = 0.02$) also revealed significant differences.

Analysis of Bcl-2 Expression. Twenty (51%) of 39 FS showed overexpression of the Bcl-2 protein, whereas none (0%) of 24 desmoid tumors showed Bcl-2 overexpression. Stratified by grade, the number of positive cases was higher in the HG-FS group (8/25) compared with the LG-FS group [12/25] of 25]. This trend of increased abnormalities in Bcl-2 expression among all of the three groups was statistically significant ($P < 0.01$). Pairwise comparison between desmoid tumors and LG-FS and between LG-FS and HG-FS showed significance only for the first pair ($P < 0.01$).

Altered Genotype and Phenotype of Ki-67, p53, pRB, and Bcl-2: Clinicopathological Implications. Molecular alterations of Ki-67, p53, pRB, and Bcl-2 in the three groups ranged from 0% in desmoid tumors to up to 93% in HG-FS, showing an increase in number with changes in tumor biology from less aggressive toward more aggressive lesions ($P < 0.01$; Table 1; Fig. 1). Coexpression of these alterations was, in general, heterogeneous, and, therefore, no direct correlation between markers was observed. Comparison of molecular data with clinicopathological variables displayed a significant association between RFS and abnormal expression of pRB in the LG-FS group ($P = 0.05$; Table 1; Fig. 2). In addition, Ki-67 overexpression was significantly predicting RFS in the subset of patients with tumors of $>5$ cm ($P = 0.02$; Table 1). No significant association was seen between RFS and p53 or Bcl-2 expression. In addition, no significant correlation was seen for abnormal expression of any of the markers and tumor site, depth of the lesion, and surgical margin status.

**DISCUSSION**

The significance of certain genetic elements for the transformation of human fibroblasts into cancer cells has recently been demonstrated *in vitro* (8). For understanding the molecular basis of clinical behavior of human fibroblastic tumors *in vivo* the knowledge of molecular alterations in malignant neoplasms compared with normal cells and intermediate lesions with the same cellular origin covering the entire spectrum of human fibroblastic neoplasms would be extremely valuable. To follow this approach, we examined the phenotypically different fibroblastic neoplasms: desmoid tumors, LG-FS, and HG-FS for abnormalities in the expression of molecular markers involved in proliferation, cell cycle control, and apoptosis by IHC and compared them with each other and with clinicopathological parameters. Normal connective tissue samples served as negative controls representing expression of the markers in healthy tissue.

Abnormalities in the expression of tumor-suppressor genes p53 and RB are the most commonly detected genetic changes in human malignant tumors (24–26). In previous studies, altered expression of p53 or pRB has frequently been demonstrated in adult soft tissue sarcomas and can be associated with poor patient outcome (9, 11, 13). In addition, overexpression of the nuclear proliferation marker Ki-67 has been shown to be frequently present and impact on prognosis of patients with soft tissue sarcomas (9, 10). Very little is known about their expression patterns in fibroblastic lesions of different biological behavior. Therefore, these markers have been selected for immunohistochemical analysis in our study. Deregulation of the balanced system of apoptosis is also a key event in carcinogenesis (14, 27), but to our knowledge, little data are available on the expression status of apoptotic regulators in fibroblastic tumors. To allow a better judgement on which pathways are significant for the phenotypic development of fibroblastic lesions, the antiapoptotic protein Bcl-2, frequently overexpressed in various other cancers (14), was included in our analysis.

Our results demonstrate a strong difference in expression of these markers between tumor entities. Desmoid tumors, except for one case with abnormal p53 expression (4%), were not different from normal connective tissue showing no abnormal expression of any of the markers in tissue fibroblasts. LG- and HG-FS displayed striking differences compared with desmoid tumors: abnormalities ranged between 20 and 56% for LG-, and 29 and 93% for HG-, malignant lesions (see “Results”). These data indicate increasing proliferative potential, irregularities in cell cycle control, and antiapoptotic regulation in malignant tumors compared with nonmalignant disease. The number of abnormalities in LG-FS was significantly less compared with HG-FS (Table 1), which reflects the accumulation of genetic alterations in more aggressive tumors. Taken together, these data support the clinical and histopathological observations about desmoid tumors and LG-FS that characterize them as nonmalignant and HG malignant lesions. The striking increase of alterations in four relevant molecular markers in HG-FS may explain their more aggressive behavior and the reduced patient survival associated with this disease (6, 7).

On the other hand, these data do not explain the clinical behavior of desmoid tumors. The expression of all of the four molecular markers in desmoids was similar to normal connective tissue. Other factors need to be analyzed to identify abnormalities that may account for desmoid tumor biology.

Associations of expression patterns of Ki-67, p53, pRB, and Bcl-2 with clinicopathological parameters revealed a significantly better

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**Table 1 Abnormal molecular marker expression in human fibroblastic tumors**

<table>
<thead>
<tr>
<th>Abnormal expression</th>
<th>Desmoid $n = 24$</th>
<th>LG-FS $n = 25$</th>
<th>HG-FS $n = 14$</th>
<th>Difference between groups (trends)</th>
<th>Correlation with recurrence-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>0/24</td>
<td>0</td>
<td>8/25</td>
<td>32</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>p53</td>
<td>1/24</td>
<td>4</td>
<td>5/25</td>
<td>20</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>pRB</td>
<td>0/24</td>
<td>0</td>
<td>14/25</td>
<td>56 ($*$)</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0/24</td>
<td>0</td>
<td>12/25</td>
<td>48</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

|                  |                 |               |               |                                   |                                          |
|                  | 12/14           | 86            |               |                                   | $P = 0.02$                               |
| p53               | 4/14            | 29            |               |                                   |                                          |
| pRB               | 13/14           | 93            |               |                                   | $P < 0.01$                               |
| Bcl-2             | 8/14            | 57            |               |                                   | $P < 0.01$                               |

* n.s., not significant.

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* $P$.
RFS (local and distant recurrence) of LG-FS patients with normal expression of pRB compared with patients with abnormal expression of pRB (Fig. 2). This could not be seen for desmoid and HG-FS patients, which was attributable to the lack of abnormalities in the desmoid tumors (0%) and the large number of abnormalities in HG-FS (93%) that did not allow any meaningful analysis of these groups. Nevertheless, the patterns of no abnormalities in nonmalignant tumors with a good prognosis and extremely high numbers of abnormalities in HG malignant tumors with a poor prognosis demonstrate the importance of pRB as a prognostic factor. This prognostic relevance of pRB is consistent with earlier observations demonstrating a survival advantage for HG sarcoma patients with normal pRB expression (13).

In addition, the previously described prognostic significance of Ki-67 for patients with HG sarcomas (10) was confirmed in our analysis. For patients with Ki-67 overexpression and tumors larger than 5 cm in diameter, regardless of tumor entity, a significant advantage \((P = 0.02)\) in RFS is demonstrated.

Molecular markers Ki-67, pRB, and Bcl-2 are uniformly negative in desmoid tumors but positive in 32–56% of LG-FS cases (Table 1). Used in combination, immunohistochemical detection of these markers may prove useful for distinction of borderline desmoid tumors and some LG-FS and, therefore, may have a role in differential pathological diagnosis of these neoplasms.

Even if these tumors are rare entities, because they cover the whole spectrum from nonmalignant to LG to HG malignant fibroblastic lesions, they may serve as a model system for the relevance of molecular factors in human soft tissue tumorigenesis or even for other cancers. Additional large-scale experiments covering a variety of molecular elements need to be conducted to determine the key factors...
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9. Lewis, J. J., Boland, P. J., Leung, D. H. Y., Woodruff, J. M., and Brennan, M. F. Elevated and absent pRB expression is associated with bladder cancer progression accounting for phenotypic differences in human fibroblastic neoplasms. This could be achieved by using the promising tissue array and DNA microarray techniques (28).

Fig. 2. Kaplan-Meier curves for RFS for local and distant recurrence of patients with desmoid tumors, LG-FS, and HG-FS. A. RFS of all of the three tumor entities including local and distant recurrence. B. RFS of LG-FS patients is significantly reduced if abnormal expression of pRB is detected (P = 0.05). C. RFS in patients with a tumor diameter of >5 cm is significantly shorter if Ki-67 is overexpressed in the lesion (P = 0.02).
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