GLI Gene Expression in Bone and Soft Tissue Sarcomas of Adult Patients Correlates with Tumor Grade

Ulrike Stein, Claudine Eder, Uwe Karsten, Wolfgang Haensch, Wolfgang Walther, and Peter M. Schlag

Max-Delbrück-Center for Molecular Medicine, [U. S., U. K., W. W.], and Charité at the Humboldt University of Berlin, Robert-Rössle Hospital and Tumor Institute, Division of Surgery and Surgical Oncology [C. E., W. H.; P. M. S.], 13125 Berlin, Germany

ABSTRACT

The GLI gene encodes a transcription factor harboring five zinc finger motifs that bind to DNA in a sequence-specific manner. The gene was originally identified because of its amplification in a human glioblastoma, and previous studies have shown it to be amplified in a significant proportion of mesenchymal tumors, such as childhood sarcomas. Here we evaluate GLI gene expression in bone and soft tissue sarcomas of adult patients. Samples from 40 patients (37 sarcomas and 3 benign mesenchymal tumors) and samples of 15 normal mesenchymal tissues were examined for GLI gene amplification and expression by Southern hybridization, reverse transcription-PCR of tissue RNA, and immunohistochemistry, using a new polyclonal GLI antibody developed against an epitope outside of the zinc finger region. In contrast to childhood sarcomas, amplification of the GLI gene was not observed in sarcomas of adult patients. Although GLI gene expression in sarcomas was significantly higher than that in normal mesenchymal tissues (P < 0.0001), the levels were very variable. Attempts to correlate the expression data with different pathophysiological parameters only showed a significant relationship to tumor grade. Based on these data, increased levels of GLI gene expression may be indicative of the aggressiveness of the tumor.

INTRODUCTION

The oncogene GLI, originally identified in a human glioma (1), represents the prototype of the GLI multigene family of DNA-binding proteins (2, 3). Homologous GLI family members have been identified in several species, such as tra-1 in Caenorhabditis elegans (4), ci in Drosophila melanogaster (5), TFIIA in Xenopus laevis (6), GLI and GLI3 in chick (7), and GLI, GL2, and GLI3 in mouse and humans (2, 3, 8). Members of this family are characterized by a conserved domain harboring five tandem cysteine-histidine (C2-H2)-type zinc fingers. Thus, it has been suggested that GLI proteins act as transcription factors by binding the zinc finger domains to the 9-bp consensus sequence GACCACCCA (9–12).

It has also been shown that GLI is a component of the Sonic hedgehog-patched signaling pathway that plays a key role in development, particularly for mesenchymal differentiation in several species (7, 8, 13–15). Other components of this pathway have been implicated to several diseases including cancer (16–19). In the case of GLI, amplification and/or overexpression have been described in brain tumors (1, 20, 21), childhood sarcomas (22, 23), skin tumors (14), and B-cell lymphomas (24). Moreover, the gene maps to the 12q13.3-q14.1 segment of human chromosome 12 (25), a region that is frequently rearranged and/or amplified in human tumors, particularly MFH,2 rhabdomyosarcoma, osteosarcoma, and liposarcoma (22, 23, 26–30). The notion that GLI may play a role in the neoplastic process is also supported by the observation that GLI protein is able to transform cells in cooperation with adenovirus E1A (31).

To determine whether the GLI gene might be connected to tumorigenesis of bone and soft tissue sarcomas derived from adult patients, we examined GLI gene amplification and the expression of the GLI gene in both the mRNA and protein levels in these tumors. Based on these data, correlation analyses with clinical features of the sarcomas such as tumor localization, tumor type, stage, and grade were performed. Most previous studies have focused on pediatric tumors, and the aim of the current study was to determine the significance of the GLI gene in sarcomas derived from adult patients. Although there was no evidence for GLI gene amplification, elevated expression of the GLI gene was observed in all the sarcomas. In addition to confirming the importance of GLI in mesenchymal differentiation, the data suggest a relationship between GLI gene expression and tumor grade.

MATERIALS AND METHODS

Patients and Tumor and Tissue Samples. Specimens from 40 patients were used in this study [37 samples of bone and soft tissue sarcomas of various histological types (MPNST, leiomyosarcomas, liposarcomas, osteosarcomas, chondrosarcomas, MFH, and others) and 3 samples of mesenchymal benign neoplasias] as well as 15 samples of normal mesenchymal tissues originating from these tumor excisions. The median age of the patients (18 females and 22 males) was 49.8 years (range, 12–87 years). Eleven of the patients were pretreated with chemotherapy, 2 with radiotherapy, and 2 patients were pretreated with combined chemotherapy/radiotherapy (Table 1). After surgery, tumor samples and the corresponding normal mesenchymal tissues were snap-frozen at −196°C, stored at −80°C, and paraffin-embedded in parallel. Grading of soft tissue sarcomas was performed in accordance with the grading system of Coindre et al. (32), and chondrosarcomas were graded according to the grading system of Unni (33). Osteosarcomas were classified with respect to their subtypes (34). For staging the tumor-node-metastasis (TNM) system was used (35).

RNA Isolation and RT-PCR. Total cellular RNA was isolated from frozen tumor samples using the miniprep-RNA protocol as described previously (36). To prove RNA quality, 5 μg of each sample were subjected to formaldehyde-agarose gel electrophoresis and stained with ethidium bromide. RT-PCR was carried out using the Invitrogen system kit (Invitrogen, Karlsruhe, Germany). The RT reaction was performed using 1 μg of miniprep-RNA and oligodeoxynucleotidic acid primers. For GLI-specific PCR, the following primer pair was used: 5'-GCTGTTGTCTCATCCTGAG3'- (sense; corresponds to the GLI cDNA sequence from 3029–3045) and 5'-GACCT-TCCTATCCTTGG-3' (antisense; corresponds to GLI cDNA from 3364–3380; Ref. 2). These GLI-specific primers yielded a 353-bp PCR product. To quantitate GLI mRNA, β-actin-specific PCR primers giving a 316-bp product were used as an internal control (37).

The RT reaction was run at 42°C for 15 min, followed by a denaturation step at 95°C for 5 min and a cooling step at 5°C for 5 min. Amplification was initially performed at 95°C for 2 min and continued for 35 cycles of denaturation (at 94°C for 30 s), annealing (at 50°C for 1 min), and extension (at 72°C for 1 min), followed by a final step at 72°C for 7 min. Under these conditions, PCR amplification was within the linear range. PCR products were separated in a 1.5% agarose gel and quantitated from video images by densitometry using the NIH Image 1.44b program. Relative GLI gene mRNA expression was calculated as the ratio of the GLI/β-actin signals.

DNA Isolation and Southern Blotting. High molecular weight DNA was isolated by phenol/chloroform extraction. Each sample (20 μg) was digested with EcoRI, electrophoresed through a 0.8% agarose gel, and blotted onto a

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1 To whom requests for reprints should be addressed, at Charité at the Humboldt University of Berlin, Robert-Rössle Hospital and Tumor Institute, Division of Surgery and Surgical Oncology, Lindenberger Weg 80, 13125 Berlin, Germany. Phone: 49-30-9417-1400; Fax: 49-30-9417-1404; E-mail: schlag@rrk-berlin.de.

2 The abbreviations used are: MFS, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; RT, reverse transcription.
noncharged nylon membrane (Qiagen GmbH, Hilden, Germany). A genomic probe for GLI (pKK36P1) was kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD; Ref. 1). Radioactive labeling was carried out by nick translation (Boehringer Mannheim GmbH, Mannheim, Germany) using α[32P]dCTP (NEN Life Sciences Products, Köln, Germany) at a specific activity of 2 × 10⁸ cpm/μg DNA. Hybridization was performed for 20 h at 68°C (38). Blots were analyzed with a Fuji BAS 2000 phosphoimager (Raytest, Straubenhardt, Germany).

**Cell Lines.** The human glioblastoma-derived cell line D259MG (39), in which the GLI gene was found to be highly (75-fold) amplified (1), was kindly provided by Dr. Darell Bigner (Duke University Medical Center, Durham, NC). Cells were grown in DMEM (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal bovine serum (Life Technologies, Inc., at 37°C and 5% CO₂). The human cell lines A-204 (rhabdomyosarcoma), provided by Dr. Darell Bigner (Duke University Medical Center, Durham, NC), KG-1 (acute myeloid leukemia), and MCF-7 and CAMA-1 (breast carcinoma) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 5 × 10⁻⁵ m mercaptopethanol at 37°C and 8% CO₂.

**Generation of Polyclonal GLI Antisera.** A 15-amino acid peptide, NH₂-EPKREREGPGIREC-CONH₂, corresponding to residues 413–427 of human GLI (S at position 427 was substituted with C) was used to raise polyclonal antibody against GLI (S at position 427 was substituted with C) was used to raise polyclonal antibody against GLI. The final bleed of rabbit SA3315 was further purified either by affinity chromatography on the peptide antigen coupled to CNBr-Sepharose (Biogenes, Berlin, Germany).

**ELISA.** Tissue culture microtiter plates were coated with 50 μl/well of a solution of 10 μg/ml of the peptides in coating buffer (pH 9.6) that was dried overnight at 37°C. The assay was performed with twofold serial dilutions of the rabbit sera and peroxidase-labeled goat antirabbit antiserum (Sifin, Berlin, Germany) and, finally, with α-phenylenediamine according to standard procedures. Preimmune sera of the animals served as controls.

**Immunohistochemistry.** Tissues used for immunohistochemistry were formalin-fixed and paraffin-embedded. Before immunohistochemistry, the paraffin sections were heated in a microwave oven for 10 min. The affinity-purified polyclonal anti-GLI antiserum was used at a dilution of 1:50. Detection of GLI protein was performed with the Vectastain Elite Kit (rabbit IgG) and the substrate 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). A mouse monoclonal antibody against the intermediate filament protein vimentin (clone V9; DAKO, Hamburg, Germany) was used as a control at a dilution of 1:50 within these tumors/tissues of mesenchymal origin. Detection was carried out with the Vectastain Elite Kit (mouse IgG). Incubations were performed in accordance with the recommendations of the manufacturer. The glioblastoma cell line D259MG served as an external positive control for expression of the GLI protein. Internal negative controls were obtained by omitting the respective primary antibodies. Evaluation of the stained slides was carried out independently by two observers (U. K. and W. H.).

For immunocytochemistry, cell lines were grown on 10-well multitest slides (Menzel-Gläser, Braunschweig, Germany) for 1–3 days. The medium was carefully removed, and the slides were air-dried. Dry slides could be stored at −80°C. For immunofluorescence, cells were briefly fixed with formalin (histology grade; Merck, Darmstadt, Germany; 5% in PBS, 5 min) and permeabilized with digitonin (Ysat, Wernigerode, Germany; 6 μg/ml in PBS, 15 min). Absorbed anti-GLI antiserum was diluted 1:200 (varied 1:100 to 1:800), and affinity-purified anti-GLI antiserum was used at 10–15 μg/ml, respectively, in culture medium. Incubations were done overnight at 4°C. Secondary antibodies (goat antirabbit; Jackson ImmunoResearch Laboritories, and rabbit antinmouse immunoglobulin antiserum; Sigma; both Cy3-labeled) were used at 1:200 in PBS and incubated for 30 min at 4°C. The slides were analyzed with an Axiosph photomicroscope (Zeiss, Jena, Germany).

**Correlation with Clinical Parameters.** To evaluate the prognostic significance of GLI gene expression, correlation analyses to a variety of clinical parameters were performed. These included the patient’s age and sex, whether or not the patient had been treated with chemotherapy and/or radiotherapy, the time interval between the detection of primary sarcoma and the diagnosis of recurrence or/and metastasis, and the histological type, localization, stage, and grade of the tumor. Statistical significance was calculated with the nonparametric Mann-Whitney rank-sum test.

**RESULTS**

**GLI mRNA Expression in Sarcomas.** The 353-bp RT-PCR product that was indicative of GLI mRNA expression was detected in all samples analyzed, including normal mesenchymal tissue, benign mesenchymal neoplasias, and all sarcomas (Fig. 1). However, there were marked differences in the levels of GLI expression, and normalization relative to β-actin revealed a 24-fold range (between relative values of 0.17 and 4.1).

As exemplified in Fig. 1, the 353-bp GLI-specific RT-PCR product was observed in MPNST (tumor 1), liposarcomas (tumors 10, 11, 12, 14, and 16), osteosarcomas (tumors 21, 22, and 23), chondrosarcomas (tumors 24 and 27), MFH (tumor 30), and a mesenchymal benign neoplasm (lipoma, tumor 38). Relative GLI expression data for all samples are summarized in Table 1. Samples of normal mesenchyme corresponding to unaffected adjacent tissue for 15 of the 37 sarcomas...
analyzed here were also subjected to RT-PCR. GLI expression levels (0.17, 0.18, 0.18, 0.21, 0.59, 0.59, 0.73, 0.73, 0.75, 0.79, 0.85, 0.86, 0.86, and 0.94; x = 0.62) were invariably found to be lower in the normal tissue than in the corresponding sarcoma.

**GLI Amplification in Sarcomas.** To determine whether GLI gene amplification may underlie the elevated expression of GLI mRNA in some samples, Southern hybridization of high molecular weight DNA was performed using the radiolabeled genomic GLI probe pKK36P1. Comparison of the GLI-specific bands showed no obvious differences in the hybridization signals between normal mesenchymal tissues and tumors or between tumors of different histopathological types.

**Immunohistochemistry of GLI Protein in Sarcomas.** Polyclonal antisera against residues 413–427 of human GLI were prepared as described in “Materials and Methods” and validated in a number of ways. Using the purified serum, distinct cytoplasmic and/or nuclear staining was observed in the GLI-positive glioblastoma-derived cell line D259MG in which GLI is amplified (Ref. 39; Fig. 2B), as well as in the rhabdomyosarcoma-derived A-204 cells. However, no staining was found in the MCF-7, CAMA-1, and KG-1 cells.

After affinity purification and absorption against human cells, the antisera was used for immunohistochemistry on sections of primary sarcomas. The results are shown in Table 1 and Fig. 2. Most sarcomas examined, 30 of 33, were found to be GLI positive by immunohistochemistry. Leiomyosarcomas, liposarcomas, MFH, osteosarcomas, and chondrosarcomas were consistently GLI positive. Two benign mesenchymal neoplasias were also marginally positive in immunohistochemistry. In both immunocytochemistry with cell lines and immunohistochemistry with tumor tissue sections, we observed a staining of the cytoplasm and/or tumor cell nuclei in varying proportions. However, the percentage of positive cells gives only one parameter for the evaluation of the protein expression level, whereas data on staining intensity were difficult to consider. As a ubiquitous mesenchymal marker, vimentin was strongly expressed in all cases.

**GLI Gene Expression in Nonmalignant and Malignant Tissues.** As indicated in Fig. 1, GLI expression varied approximately 24-fold among different tumor types and in normal mesenchymal tissue. We therefore looked for trends in the relative expression levels in normal mesenchymal tissues (15 samples), mesenchymal benign neoplasias (3 samples), primary sarcomas (12 samples), local recurrent sarcomas (17 samples), and sarcoma metastases (8 samples). As shown in Fig. 3, no significant difference was found within the group of nonmalignant tissues (normal tissues versus mesenchymal benign neoplasias) or within the group of malignant tumors (primary sarcomas versus recurrent or metastastic-stage tumors). However, when comparing nonmalignant (normal tissues plus mesenchymal benign neoplasias) versus sarcomatous tissue (primary sarcomas plus recurrences plus metastases), a statistical difference was found (P < 0.0001). This also holds true when comparing the nonmalignant group versus primary malignancy.
Fig. 2. GLI-specific immunohistochemistry of bone and soft tissue sarcomas. The glioblastoma-derived cell line D259MG served as an external positive control. Vimentin staining with antibody V9 was performed in all cases to verify the mesenchymal origin of the analyzed tumors. A-C, cell line D259MG; D-F, liposarcoma (multiform, tumor 17); G, liposarcoma (pleomorphic, tumor 19); H, osteosarcoma (tumor 21); I, MFH (tumor 28). A and D, negative control (without primary antibody); B, E, and G-I, GLI-specific antiserum, affinity-purified; C and F, vimentin (V9). Magnification, ×400. Immunohistochemistry data for all samples are summarized in Table 1.
findings with a panel of clinical and histopathological parameters (Table 1). GLI expression was detected at the RNA level in all analyzed malignant tumor samples (37 of 37), although its amount varied within a large range (24-fold). GLI protein expression in sarcomas was also frequently observed by immunohistochemistry using a newly developed and characterized polyclonal GLI-specific antibody toward a non-zinc finger domain. GLI mRNA expression has already been reported for other human tumors, such as tumors of the central nervous system (40), and elevated levels have been observed infrequently in some childhood sarcomas (22, 23) as well as in meningiomas and astrocytomas (20).

In normal tissues of the adult, human GLI gene expression has also been demonstrated in the testis, fallopian tube, and myometrium (2), supporting our findings of GLI expression in all 15 normal mesenchymal tissue samples. Interestingly, comparison of the GLI gene expression levels of normal mesenchymal tissue with those of the corresponding sarcoma obtained from the same patient revealed that in every case, the level of GLI gene expression is lower in the normal tissue than it is in the tumor.

Amplification of the GLI gene was not detectable within the panel of sarcomas from the adult patients analyzed in this study. These findings are consistent with those described for leiomyosarcomas (41) and pediatric brain tumors (42), but contrast with the albeit rare amplifications of GLI reported in childhood sarcomas (22, 23), squamous cell carcinomas (43), and gliomas (44).

**DISCUSSION**

To evaluate whether the GLI gene might serve as a molecular marker for human bone and soft tissue sarcomas, we examined its expression and amplification in sarcomas of adults and correlated the

![Fig. 3. GLI gene expression in nonmalignant and malignant tissues.](image-url)

When comparing the two groups of nonmalignant tissues (normal mesenchymal tissues (15 samples) plus mesenchymal benign neoplasias (3 samples)) versus malignant tumors (primary sarcomas (12 samples) plus local recurrent sarcomas (17 samples) plus sarcoma metastases (8 samples)), a statistically significant correlation was found with the higher GLI expression levels in the malignant group ($P < 0.0001$). Comparison of the nonmalignant group versus primary sarcomas alone ($P = 0.0013$), versus recurrences alone ($P = 0.0270$), or versus metastases alone ($P < 0.0001$) also revealed significant correlations. No significant differences were found within the group of nonmalignant tissues or within the group of malignant tumors.

**GLI Gene Expression and Grade of Sarcomas.** We also performed correlation analyses of GLI gene expression with several clinical parameters, but we found no significant correlations with the patient’s age or sex, tumor localization, tumor size, preoperative chemotherapy and/or radiotherapy, time to recurrence, and metastases-free survival time. However, there was a clear relationship between GLI expression and tumor grade (Fig. 4), with an average GLI expression of $x = 0.73$ for tumor grade 1 (13 samples), $x = 1.16$ for tumor grade 2 (8 samples), and $x = 1.92$ for grade 3 (16 samples). Significant differences were found when comparing the GLI expression of grade 1 tumors with that of grade 2 tumors ($P = 0.0125$), of grade 2 tumors with that of grade 3 tumors ($P = 0.0046$), and of grade 1 tumors with that of grade 3 tumors ($P < 0.0001$).

![Fig. 4. GLI gene expression in correlation to tumor grade.](image-url)

When comparing GLI expression with the tumor grade, statistically significant differences were found with the higher GLI expression data in high-grade sarcomas: grade 1 tumors versus grade 2 tumors ($P = 0.0125$), grade 2 tumors versus grade 3 tumors ($P = 0.0046$), and grade 1 tumors versus grade 3 tumors ($P < 0.0001$). Average GLI expression was as follows: grade 1 tumors (13 samples), $x = 0.73$; grade 2 tumors (8 samples), $x = 1.16$; and grade 3 tumors (16 samples), $x = 1.92$. 

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The most significant findings were the elevated GLI expression in sarcomas compared with nonmalignant mesenchymal tissues and the correlation of GLI expression with the grade of the sarcomas. We conclude that GLI gene expression is increased in cells of mesenchymal origin with differentiated phenotypes. Our data are in accordance with the observation reported for childhood sarcomas that GLI expression was restricted to poorly differentiated tumors (22). Very recently, overexpression of the GLI gene in comparison to the surrounding normal tissue has been reported for rhabdomyosarcomas in mice (45). Moreover, the involvement of GLI in the signaling pathway of the development of rhabdomyosarcomas has been described previously (46). Our finding that the tumor grade directly correlates with the expression of GLI supports the hypothesis that the GLI gene may play a role in the malignant transformation of mesenchymal cells.

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REFERENCES


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