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Short Communication

Single Somatic *ras* Gene Point Mutation in Soft Tissue Malignant Fibrous Histiocytomas

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The frequency of* ras *gene mutations in human soft tissue malignant fibrous histiocytomas within and around the hot spot codons (12, 13, and 61) of all* ras *genes, (H-*ras*-1, K-*ras*-2, and N-*ras*) was studied by nested polymerase chain reaction and direct DNA sequencing from archival formalin-fixed, paraffin-embedded tissue. Light microscopy and immunohistochemistry served to define malignant fibrous histiocytoma. All of the four differentiation subtypes (storiform-pleomorphic, inflammatory, myxoid, and giant cell) were investigated. Nine of thirty-two malignant fibrous histiocytomas (28%) contained* ras *gene point mutations. The highest incidence was found in the myxoid subtype (four of nine). H-*ras*-1 gene codon 12.2 was the only codon affected and contained in all mutated cases a GGC→GTC exchange. Seven of the nine mutations were homozygous and probably affected more than 80% of the tumor DNA. The flanking regions of all hotspot codons did not contain any point mutation. The presence of a single and often homozygous point mutation of the H-*ras*-1 gene, especially in myxoid malignant fibrous histiocytoma could serve as a basis for further genomic discrimination of myxoid sarcomas. (Am J Pathol 1996, 148:731–738)

The *ras* genes are the most frequently found activating oncogenes in human tumors. They gain their oncogenic activity by a single amino acid substitution in the hot spot positions 12, 13, and 61 of the H-*ras*-1, K-*ras*-2, or N-*ras*-encoded p21 protein.[1,2] The Ras protein-mediated signal transduction pathway has turned out to be at the heart of growth control in higher eukaryotic organisms. It is so highly conserved that its components are functionally interchangeable between mammals, flies, and worms.[3] The biochemistry of the Ras signal transduction pathway was recently characterized in more detail; intracellular Grb2-Sos (Grb 2, mammalian homologue of sem-5 gene, that encodes a protein with Src-homology 2 and Src-homology 3 domains; Sos, protein encoded by the son of sevenless gene) complexes bind to Ras proteins and induce a cascade of kinases to send trophic signals to the nucleus.[3] Mutated Ras proteins promote constitutive activation of its downstream effector pathways.[4]

Activated *ras* genes were primarily detected in transfection experiments and turned out to induce malignant mesenchymal tumors (erythroleukemia and sarcomas) in rodents.[5,6] Light microscopy, histochemistry, and electron microscopy of the sarcomas suggested a histiocyte/monocyte/macrophage origin.[7–9] Contrary to these experimental data, activated *ras* genes in humans were mostly investigated in carcinomas. They were found predominantly in adenocarcinomas of the pancreatic/bilary region, colon, and lung.[2] Although *ras* mutations were not clearly associated with specific clinical and biological features, their appearance turned out to be associated with poor prognosis in certain tumors (eg, lung cancer and colon cancer).[10–12] Sufficient data

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Table 1. *Patients, Tumor Data, and Results of* ras *Gene Direct Sequencing of Soft Tissue MFHs*

Case	Age/Sex	Site/Localization	Mutation
SP 1	46/M	Thigh/d	
SP 2	83/M	Shoulder/s	
SP 3	70/M	Upper arm/s	
SP 4	45/M	Upper arm/s	
SP 5	66/M	Thoracic wall/s	
SP 6	92/F	Forearm/d	
SP 7	55/M	Upper arm/s	H-*ras*-1, codon12 GGC→GTC/het
SP 8	61/F	Thigh/d	
SP 9	51/F	Thoracic wall/d	
SP 10	73/M	Thigh/d	H-*ras*-1, codon12 GGC→GTC/hom
SP 11	52/M	Ear/s	
SP 12	70/M	Thoracic wall/s	
MYX 1	46/F	Thigh/d	H-*ras*-1, codon12 GGC→GTC/hom
MYX 2	65/M	Lower leg/s	
MYX 3	66/F	Thigh/s	
MYX 4	48/M	Thigh/d	H-*ras*-1, codon12 GGC→GTC/hom
MYX 5	68/F	Upper arm/s	H-*ras*-1, codon12 GGC→GTC/hom
MYX 6	56/F	Retroperitoneum/d	
MYX 7	59/F	Retroperitoneum/d	H-*ras*-1, codon12 GGC→GTC/hom
MYX 8	54/M	Thigh/s	
MYX 9	59/M	Thoracic wall/d	
XAN 1	58/F	Axilla/s	
XAN 2	51/F	Thoracic wall/d	H-*ras*-1, codon12 GGC→GTC/het
XAN 3	48/F	Retroperitoneum/d	
XAN 4	77/F	Thigh/d	
XAN 5	60/F	Axilla/d	
XAN 6	75/F	Neck/d	
GC 1	52/M	Thigh/d	H-*ras*-1, codon12 GGC→GTC/hom
GC 2	81/F	Thoracic wall/d	
GC 3	68/F	Thigh/d	
GC 4	69/M	Elbow/s	H-*ras*-1, codon12 GGC→GTC/hom
GC 5	52/F	Retroauricular/s	

SP, storiform-pleomorphic MFH; MYX, myxoid MFH; XAN, xanthomatous MFH; GC, giant cell MFH; s, superficial; d, deep; het, heterozygous; hom, homozygous.

on *ras* gene activation in human sarcomas are currently missing. In this paper we report sequence analysis data of all the hot spot sequences of all three *ras* genes of the most frequently diagnosed human soft tissue sarcoma, malignant fibrous histiocytoma (MFH).

To investigate a possible relationship between *ras* gene activation and tumor differentiation we searched for *ras* gene mutations in all of the four typical MFH differentiation subtypes (storiform-pleomorphic, xanthomatous (inflammatory), myxoid, and giant cell). The *ras* gene DNA was amplified by polymerase chain reaction (PCR) and directly sequenced from archival formalin-fixed, paraffin-embedded tissue.

Materials and Methods

Patients and Tissue Specimens

Tumors from 32 patients (15 male, 17 female) were surgically removed, fixed in formalin, and sent to the Department of Pathology of the Giessen University or Jena University for histological diagnosis. Of these patients, 14 had superficial (subcutaneous) and 18 had deep (fascial, intramuscular, or visceral) disease. Tumor sizes ranged between 1.2 and 20 cm. After routine embedding, light microscopy as well as immunohistochemistry led to the final diagnosis of MFH. All cases were reviewed, and tissue blocks with definite differentiation according to the current classification[13] were selected. For grading, the National Cancer Institute system was used.[13] Only tissue sections that contained more than 90% tumor tissue were used for the preparation of DNA. Table 1 shows a list of the analyzed cases.

Immunohistochemistry

The antibodies are described in Table 2. Immunohistochemical staining was performed using the alkaline phosphatase anti-alkaline phosphatase method.[14] Incubation times, link antibody concentrations, and development were as previously described.[15]

Table 2. *Monoclonal Antibodies Used for Immunohistochemical Staining*

Antigen	Antibody (code)	Working dilution	Source
Vimentin	Monoclonal (V9)	1:50	DAKO (Glostrup, Denmark)
Cytokeratin	Monoclonal (KL1)	1:100	Dianova (Hamburg, Germany)
Desmin	Monoclonal (D33)	1:100	DAKO
Muscle actin	Monoclonal (HHF35)	1:50	Enzo (New York, NY)
Endothelium	Monoclonal (BMA120)	1:10	Behring (Marburg, Germany)
Cow S-100	Polyclonal	1:2000	DAKO
KiM1p (CD68)	Monoclonal	1:1000	R. Parwaresch (Kiel, Germany)
Factor XIIIa	Polyclonal	1:50	Behring

Purification of Genomic DNA

For the detection of *ras* mutations, genomic DNA was extracted from sections cut from the paraffin-embedded tissue blocks following the procedure described by Heller et al.[16] The following cell lines with known *ras* mutations were used as positive controls for establishing the sequencing method in the beginning of the investigation: T24 (c-H-*ras*, codon 12 GGC→GTC homozygous), RL95–2 (c-H-*ras*, codon 61 CAG→CAT heterozygous), Molt 4 (N-*ras*, codon 12 GGT→TGT heterozygous), and HT 1080 (N-*ras*, codon 61 CAA→AAA heterozygous). DNA was extracted by proteinase K digestion. Cross-contaminations of patient samples with cell-line DNA were excluded as the cells were not extracted in the room where patient samples were handled. Cell-line DNA was used only in the first phase of the investigation when the sequencing method had yet to be established. It was never amplified in one run together with patient material.

Polymerase Chain Reaction

PCR was performed as described previously[17] with the following modifications: for the first 35 cycles and second 25 cycles, annealing was at 58°C for 60 seconds and extension was at 72°C for 75 seconds (time extension 3 seconds/cycle). To enable the purification of PCR products by magnetic beads, the internal downstream primer was labeled with biotin. PCR was performed in a Robotherm thermal cycler (Bühler, Freiburg, Germany). Primers (Table 3) were commercially obtained (Roth, Karlsruhe, Germany). To achieve maximal sensitivity, a nested PCR protocol was used.[18] PCR products were analyzed on a 2.5% agarose gel and visualized by ethidium bro-

Table 3. *Sequences and Positions of PCR Primers*

Primer	Nucleotide sequence	Position
H 12/13 ext up	5′GGAGACCCTGTAGGAGGACCC	1625–1645
H 12/13 ext down	5′TCTATAGTGGGGTCGTATTCGTCC	1779–1756
H 12/13 int up	5′GCAGGCCCCTGAGGAGCGATG	1652–1672
H 12/13 int down	B-5′AAAATGGTTCTGGATCAGCTGGATG	1753–1729
H 61 ext up	5′GCAGGATTCCTACCGCAAGCAG	2045–2066
H 61 ext down	5′TCAAAAGACTTGGTGTTGTTGATGG	2209–2185
H 61 int up	5′TCATTGATGGGGAGACGTGCCTG	2071–2093
H 61 int down	B-5′CACAGGAAGCCCTCCCCGGTG	2176–2156
K 12/13 ext up	5′TTATGTGTGACATGTTCTAATATAGTC	6314–6340
K 12/13 ext down	5′AAACAAGATTTACCTCTATTGTTGGATC	6500–6473
K 12/13 int up	5′ATTATAAGGCCTGCTGAAAATGACTG	6358–6383
K 12/13 int down	B-5′GTCCACAAAATGATTCTGAATTAGCTG	6466–6440
K 61 ext up	5′CTCAGGATTCCTACAGGAAGCAAG	19462–19485
K 61 ext down	5′TATCTTCAAATGATTTAGTATTATTTATGG	19632–19603
K 61 int up	5′AGTAATTGATGGAGAAACCTGTCTC	19487–19511
K 61 int down	B-5′AAGAAAGCCCTCCCCAGTCCTC	19592–19571
N 12/13 ext up	5′CAGGTTCTTGCTGGTGTGAAATGAC	1–24
N 12/13 ext down	5′TCTATGGTGGGATCATATTCATCTAC	130–105
N 12/13 int up	5′TGACTGAGTACAAACTGGTGGTGG	22–44
N 12/13 int down	B-5′AAGTGGTTCTGGATTAGCTGGATTG	103–79
N 61 ext up	5′ATTCTTACAGAAAACAAGTGGTTATAG	2–28
N 61 ext down	5′CGCAAATGACTTGCTATTATTGATGG	162–137
N 61 int up	5′GGTGAAACCTGTTTGTTGGACATAC	31–55
N 61 int down	B-5′GAGGAAGCCTTCGCCTGTCCTC	126–105

Sequences were taken from Capon et al[36] (H-*ras*-1), McGrath et al[37] (K-*ras*-2), and GeneBank, Accession L00040 K00082 and L00041 K00082 (N-*ras*). Int, internal nested PCR primer; ext, external nested PCR primer. B indicates a biotin label at the 5′ end to enable solid phase direct sequencing.

Table 4. *Sequencing Primers*

Primer	Sequencing direction	Nucleotide sequence	Position
H 12/13	Reverse	5′AATGGTTCTGGATCAGCTG	1751–1733
H 61	Reverse	5′GAAGCCCTCCCCGGTG	2171–2156
K 12/13	Forward	5′GGCCTGCTGAAAATGACTG	6365–6382
K 61	Forward	5′TGATGGAGAAACCTGTCTC	19493–19511
N 12/13	Reverse	5′AGTGGTTCTGGATTAGCTG	102–84
N 61	Forward	5′GGTGAAACCTGTTTGTTGG	31–49

Sequences were taken from Capon et al[36] (H-*ras*-1), McGrath et al[37] (K-*ras*-2), and GeneBank, Accession L00040 K00082 and L00041 K00082 (N-*ras*).

mide staining. To avoid contaminations, the suggestions of Kwok[19] were meticulously obeyed.

Direct DNA Sequencing

Template preparation was carried out using Dynabeads M-280 Streptavidin as described by Hultman et al.[20] DNA sequencing was performed according to a modified Sanger dideoxy chain termination protocol, using an Applied Biosystems (Foster City, CA) Prism T7 terminator single-stranded DNA sequencing kit with fluorescence-labeled chain terminators. Either the biotinylated strand bound to the solid phase or the precipitated strand in the supernatant was used as a sequencing template. To avoid sequencing over GC-rich regions upstream from both hot spots in H-*ras*-1 and codon 12/13 in N-*ras*, these fragments were sequenced in the reverse direction. K-*ras*-2 and the fragment containing N-*ras* codon 61 did not pose such problems and were therefore sequenced in the forward direction. In detail, the purified single-stranded DNA from 40 μl of PCR mixture was resuspended in 15 μl of Tris-ethanolamine buffer, and 1 pmol of sequencing primer (see Table 4 for sequences) and 5 μl of 5X reaction buffer were added. The primer annealing took place in a cryostat waterbath, and after a 2-minute incubation at 65°C, the samples were slowly cooled to 37°C over a period of 30 minutes. After annealing, 4 μl of terminator mix and 1.5 U of Sequenase were added. Chain extension/termination took place during a 10-minute incubation in a 37°C waterbath. All reactions were separated and analyzed on an Applied Biosystems 373A automated sequencer. To detect point mutations, the chromatograms were visually screened for the existence of overlapping peaks independently by two investigators (R. M. B. and S. B.). To assess the validity of an overlap, ie, a heterozygous mutation, the height of the peak overlapping the wild-type sequence was compared with the intermediate height of this base's peaks in the vicinity of the hot spot. An overlap was considered to represent a mutation only if it was at least one-fifth as high as the neighboring peaks representing the base in question. To confirm the mutations, new PCR reactions were prepared and sequenced as described above. A dilution series of the homozygously mutated cell line T24 (H-*ras*-1 codon 12 GTC) with human placenta DNA showed that for the detection of a heterozygous mutation the mutated allele has to constitute at least 20% of the template DNA (data not shown).

Statistical Analysis

The clinicopathological variables were cross-tabulated with source (MFHs with and without *ras* gene mutations), and significance of associations was determined using Fisher's exact test. For age and tumor size statistics the Mann-Whitney U test was used. A P value of ≤ 0.05 was considered statistically significant.

Results

Histological Findings and Immunohistochemical Findings

All 32 tumors were reviewed before *ras* gene analysis. According to World Health Organization criteria they were classified as MFH of storiform-pleomorphic type, xanthomatous (inflammatory) type, myxoid type, or giant cell type (Figure 1). As diagnostic criteria of MFHs are currently in discussion,[21] immunohistochemistry served to exclude pleomorphic sarcomas and spindle cell sarcomas of another histogenetic origin. We made sure that the tumors did not show desmin, BMA 120, or protein S 100 expression. A weak cytokeratin expression in a minority of tumor cells (<5%) was considered to be consistent with the MFH diagnosis. Some of the tumor cells showed a weak cytoplasmic muscle actin expression, which was interpreted as a partial myofibroblastic phenotype. Most of the tumors showed KiM1p and factor XIIIa expression. Vimentin expres-

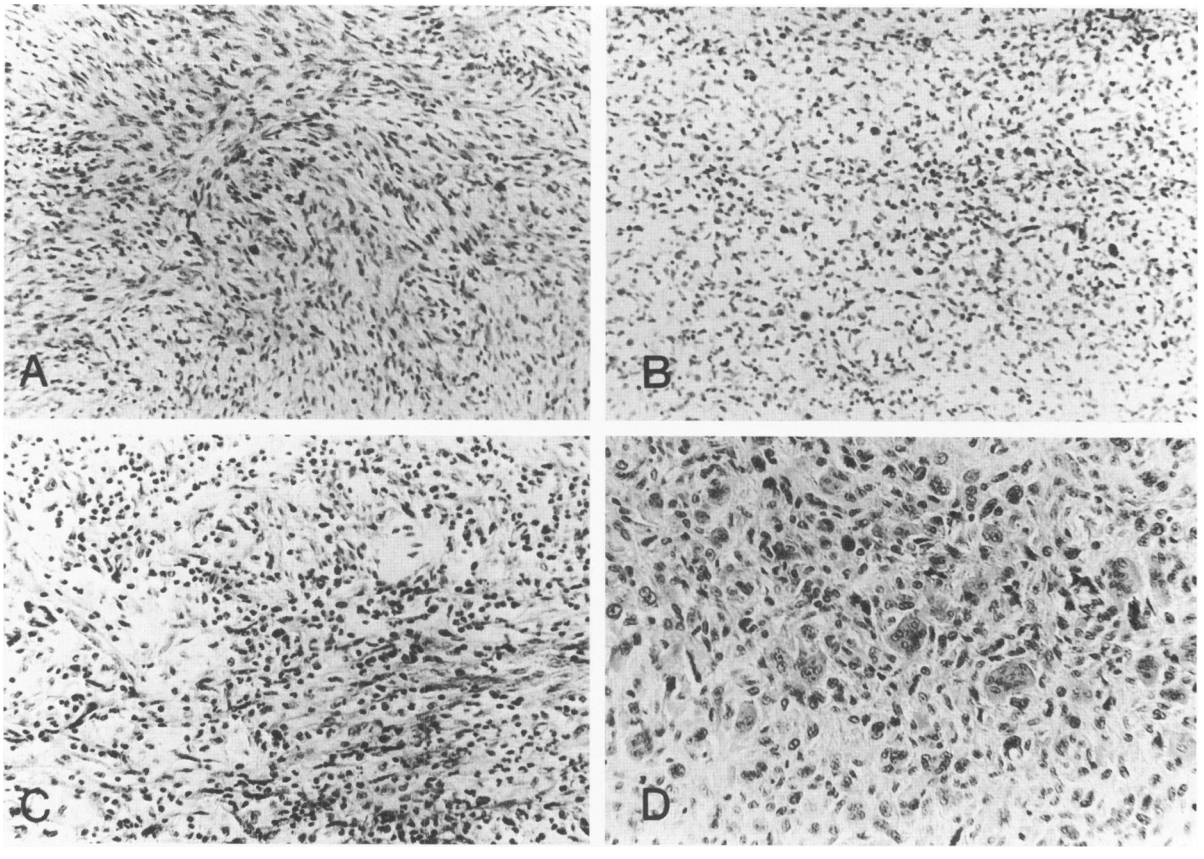

Figure 1. *Investigated tumor examples: storiform-pleomorphic* (A), *myxoid* (B), *xanthomatous (inflammatory)* (C), *and giant cell* (D) *soft tissue MFH. H&E original magnification, ×16.*

sion served as a quality control marker of the overall immunoreactivity of the tumor tissue.

Molecular Analysis

All samples yielded PCR amplification products for all fragments, indicating adequate DNA preparation. The automated sequencing method allowed us to analyze the hot spot regions (codons 12, 13, and 61) of all three *ras* genes in each of the investigated cases. Additionally, 10 to 30 bases upstream and downstream of the hot spots were of sufficient quality for evaluation. In the primary and repeated analysis we detected *ras* gene point mutations in 9 of the 32 MFHs (28%). Surprisingly, only one type of point mutation in position 2 of codon 12 in the H-*ras*-1 gene could be detected, it was a GGC→GTC transition (Gly12Val).

In 7 of 9 mutated tumors this point mutation turned out to be homozygous. One mutation detected in a xanthomatous (inflammatory) MFH and one of the two mutations detected in storiform-pleomorphic MFHs showed heterozygous sequencing results. None of the other MFHs contained point mutations in codon 12, 13, or 61 of the K-*ras*-2 and N-*ras* gene, in codon 13 or 61 of the H-*ras*-1 gene, or in the vicinity of these hot spots. The most prominent accumulation of the H-*ras*-1 codon 12 mutation occurred in the myxoid subtype of MFH with a 44% incidence (4 of 9). In contrast, all of the other histological subtypes displayed only four such mutations: 2 of 12 (17%) in the storiform-pleomorphic group, 1 of 6 (17%) in xanthomatous (inflammatory) MFHs, and 2 of 5 (40%) in the giant cell category.

MFHs with *ras* gene point mutations did not significantly differ from MFHs without mutations with respect to patients' age, sex, tumor size, tumor localization (superficial *versus* deep), grading, and primary tumor classification.

Discussion

The Ras protein p21 gains its oncogenic potential by activating point mutations of the H-*ras*-1 (chromosome 11p15), K-*ras*-2 (chromosome 12p12), or N-*ras* (chromosome 1p22-p32) genes at the so-called hot spot codons 12, 13, and 61. H-*ras*-2 (X chromosome)

and K-*ras*-1 (chromosome 6p12-p32) are *ras* pseudogenes, which do not participate in this process.[22] The frequency of *ras* gene mutations ranges between 15 and 30% of all human neoplasms.[1,2,23,24] Although it is not definitely clear which *ras* gene is active in certain human tissues, K-*ras*-2 mutations can be found predominantly in adenocarcinomas (lung, colon, biliary tract, and pancreas), whereas H-*ras*-1 activation is associated with human bladder carcinomas, and leukemic/lymphoid neoplasms may contain N-*ras* mutations. However, also within the same organ, eg, pancreas, the frequency of *ras* gene mutations varies considerably among the different subtypes of these tumors, from 6% in acinar carcinomas to 90% in ductal carcinomas.[25,26]

Considering the most frequent soft tissue sarcoma of adults, the MFH with its four subtypes, only very few *ras* gene analyses are known. Bos et al,[2] using oligonucleotide hybridization, reported that none of 10 MFHs contained any *ras* gene mutation. Gill et al,[27] using an NIH-3T3 DNA transfection assay, could not record any transforming activity in three MFHs. Recently, Wilke et al,[28] who sequenced H-*ras*-1, reported codon 12 point mutations in 2 of 6 MFHs without giving information about the tumor subtypes.

To perform a complete analysis of *ras* gene hot spots we sequenced the H-*ras*-1, K-*ras*-2, and N-*ras* genes of 32 MFHs of all four subtypes. Immunohistochemistry excluded other sarcomas with similar phenotype. To avoid amplification of *ras* pseudogenes, external nested PCR primers were used that were complementary to parts of the H-*ras*-1 and K-*ras*-2 intron flanking regions.

The most noteworthy finding of our study was the exclusive involvement of the H-*ras*-1 gene codon 12.2 mutation (GGC→GTC) as the only *ras* gene mutation found in 9 of the 32 (28%) MFHs. H-*ras*-1 codons 13 and 61, K-*ras*-2 codons 12, 13, and 61, and N-*ras* codons 12, 13, and 61 as well as the flanking regions of these codons did not contain any *ras* gene mutation. Compared with the more common types of malignant human tumors that carry *ras* gene mutations frequently (eg, pancreatic cancer, colon cancer, and adenocarcinoma of the lung), this constellation can be considered unique, as they usually contain *ras* gene mutations within more than one single codon position (eg, pancreatic cancer at codon 12.1, 12.2, or 12.3).[4] Thus, H-*ras*-1 codon 12 is likely to be the *ras* gene mutation preselection site of MFHs.

From the methodological point of view, the fact that seven of the nine H-*ras*-1 mutations found in paraffin-extracted DNA were homozygous implies that a large number of tumor cells carry this mutation. Our dilution experiments suggest that within a heterogeneous DNA source (like tumor DNA containing DNA from tumor cells themselves, stromal cells, and inflammatory cells) a minimum of 20% mutated or nonmutated cells is necessary for sensitive detection of the genomic type. For this reason, our detection of homozygous mutations in MFHs means that most probably more than 80% of the tumor cells contain the activated *ras* gene on both alleles. On the other hand it is clear that *ras* gene mutations in the xanthomatous (inflammatory) MFH can be underestimated. The large number of inflammatory cells (often more than 30 to 40% of cells within the tumor) will yield wild-type sequencing signals and might lead to a false heterozygous gene type on PCR-based DNA sequencing. Although it is very likely that many MFH tumor cells carry H-*ras*-1 mutations, our results do not suggest how H-*ras* was mutated or whether mutations are important for the development of MFHs. Additional analysis of small primary tumors and their advanced recurrences or of fibrohistiocytic tumors with an intermediate malignancy grade might give additional information with respect to their appearance in MFH development.

Biochemical data indicate that the GGC→GTC exchange detected results in an activation of the signal transduction pathway. It leads to an amino acid substitution of the wild-type Gly12 to Val12, which accounts biochemically for a 93% reduction of the GTPase dissociation rate.[29] Thus, it is possible that the H-*ras*-1 point mutations in soft tissue MFHs possess a strong biological activity. As basic clinicopathological data analyzed did not differ between point-mutation-carrying and -noncarrying MFHs, additional analysis of prognostic relevance of *ras* gene mutations in MFHs is necessary.

Another remarkable result of the study was the inhomogeneous distribution of mutations among the four subtypes of MFH with a 44% maximum of the H-*ras*-1 mutation in the myxoid type. Although distribution of *ras* gene mutations among the four types was not statistically significant, this constellation may point to a phenotype-related appearance of activated *ras* genes in sarcomas of MFH type, as is known from some carcinomas[25,26] and pheochromocytoma cell lines.[30] Additional studies of liposarcomas (n = 24), malignant peripheral nerve sheath tumors (n = 11), leiomyosarcomas (n = 10), and fibrosarcomas (n = 6) showed that these sarcomas do not contain H-*ras*-1 codon 12.2 mutations.[31]

Human MFH H-*ras*-1 sequencing data from Wilke et al[28] underline the involvement of H-*ras*-1 codon 12.2. In their two cases a base exchange,

GTC→GAC, results in an amino acid exchange from glycine to aspartic acid. Although the site and biochemical activity of both point mutations is equivalent,[32] we do not have a reasonable explanation for this difference between mid-European and American MFHs.

Recent experimental data from transgenic mice underline the capacity of (promoter-mediated) *ras* gene activation and MFH-like phenotype.[33] A possible link between genotype and phenotype alterations might exist with respect to the intermediate filament network, as the activated *ras* genes are suspected to induce changes in actin cytoskeleton[34] and to inhibit myogenesis[35] *in vitro.* Thus, speculation might arise whether some MFHs represent genetically inhibited myogenic sarcomas.

In summary, a single point mutation, H-*ras*-1 codon 12.2, among all hot spot regions of the three *ras* genes in adult soft tissue MFHs could be detected. It could serve as the basis for additional *ras* gene analysis in adult soft tissue sarcomas and might be a differential diagnostic criterion of approximately one-fourth of the MFHs. There seems to be no association of H-*ras*-1 point mutations with basic primary tumor parameters. Additional analysis of clinicopathological data is required for the determination of the biological significance and prognostic impact of H-*ras*-1 Gly12Val mutations in adult soft tissue MFHs.

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