

Papers

Malignant fibrous histiocytomas and H-ras-1 oncogene point mutations

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Abstract

Aims—To investigate the types and the frequencies of H-ras-1 gene mutations in malignant fibrous histiocytomas.

Methods—Thirty five samples of malignant fibrous histiocytoma tissue were searched for point mutations within “hot spot” codons 12 and 13 of the H-ras-1 oncogene by the specific “nested” polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) and a direct cycle sequencing procedure.

Results—In contrast to previous reports, none of the tumours contained a point mutation or any other changes within or around the hot spot gene sequences.

Conclusions—These data indicate that H-ras-1 oncogenic activation is not required in the molecular pathway of malignant fibrous histiocytoma formation and cannot be used as a discriminating factor for diagnostic sarcoma typing.

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Keywords: malignant fibrous histiocytoma; restriction fragments length polymorphism; H-ras-1 oncogene

Cytogenetic and molecular studies have greatly improved our understanding of the role of different chromosomal translocations specific for soft tissue sarcomas and have given clues to the functions of many genes involved in their tumorigenesis. The role of certain oncogenes and tumour suppressor genes in the evolution and progression of soft tissue sarcomas has also been determined.¹⁻⁵ Soft tissue tumour research has included the determination of structural and functional changes seen in well known families of proto-oncogenes—for example, the ras gene family.^{6,7}

The ras proto-oncogene family (H-ras-1, K-ras-2, and N-ras genes) encodes a specific class of highly conserved G proteins (21 kDa in size), which exhibit GTPase and GTP/GDP binding activities. These proteins are involved in the transduction of external signals from the cell surface to the nucleus. Wild-type Ras protein (p21) is activated by GTP binding; it becomes inactive after conversion of GTP to GDP. Structurally altered p21 cannot become inactivated; such conformational changes are caused by point mutations in H-ras, K-ras, and N-ras, which have been identified in a wide variety of

human cancers.⁸⁻¹⁰ How these mutations produce their effect remains unclear, but it is noteworthy that the sites for mutations within ras genes are limited to codons 12, 13, and 61, which are located within the regions coding for GTP/GDP binding.¹¹ The frequency of ras mutations within different types of neoplasm is estimated, on average, to be 15-30%,^{8,11} but varies greatly between tumours of different types, being < 5% in Hodgkin's disease or neuroblastoma,^{12,13} 37% in endometrial carcinoma,¹⁴ and almost 90% in pancreatic cancer.¹⁵ However, although ras proto-oncogene mutations are involved in tumorigenesis, they do not seem to be sufficient or specific for any type of neoplastic process; their appearance has been connected with poor prognosis in some tumours, mainly lung and colonic cancers.¹⁶⁻¹⁸ The accumulation of additional DNA defects, detected in all types of tumours, is mandatory for malignant transformation.^{19,20}

The data concerning ras oncogene mutations in soft tissue and bone sarcomas are limited to a few reports.^{6,7,21-23} Bohle *et al* have demonstrated a single somatic H-ras-1 gene point mutation in a high fraction of malignant fibrous histiocytomas, particularly in the myxoid subtype (30% of tumours).²³ Codon 12 of the H-ras-1 gene was the only one affected and contained the GGC→GTC (Gly→Val) transversion in all mutated cases. Most of these mutations were homozygous and affected more than 80% of the tumour cells. The authors suggested that the presence of a single and often homozygous point mutation within the H-ras-1 gene was a specific feature of myxoid malignant fibrous histiocytomas, and could be used as a further genomic marker and discrimination factor of sarcomas.

The aim of our work was to investigate further the types and the frequencies of H-ras-1 gene mutations in malignant fibrous histiocytomas using a simple and sensitive polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique and direct sequencing method.

Material and methods

There were 104 patients with different types of soft tissue sarcomas treated in the Regional Center of Oncology in Lodz from 1991 to 1997. The 115 formalin fixed, paraffin wax embedded samples from all analysed tumour lesions were diagnosed in the department of

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tumor pathology, Medical University of Lodz. The grade of malignancy was determined according to the FNCLCC method.²⁴ The histological diagnosis was completed according to the criteria of Enzinger and Weiss.²⁵ The slides were reviewed by three pathologists (AS; and B Haraczga-Bal and B Olborski). Immunohistochemical staining for vimentin, desmin, endothelium, smooth muscle actin, S-100 protein, HMB-45, CD68, and CD34 antigens was used to confirm the diagnosis. All patients with malignant fibrous histiocytomas were selected for our study. There were 35 patients: 14 women and 21 men (age range, 30–90 years; mean, 58). Most of the tumours were primary tumours (n = 31), whereas the remaining lesions were recurrences (n = 4). Malignant fibrous histiocytomas were subclassified according to the predominant pattern. Ten tumours were of the myxoid subtype and the remaining tumours were of the storiform-pleomorphic subtype (25 specimens).

Tumour tissue was selected to contain as little normal tissue as possible. We isolated DNA by a routine proteinase K digestion and phenol/chloroform extraction procedure. DNA isolated from the T24 cell line, which has a homozygous mutation of codon 12 of the H-ras gene (GGC→GTC) was used as a positive control for further analysis.

A “nested” PCR was performed for the amplification of H-ras codons 12/13, according to Bohle *et al.*²³ The procedure was carried out in a final volume of 20 µl in a Perkin Elmer 2400 thermocycler. DNA template (50 ng) was added to the reaction mixture containing 0.5 U Taq DNA polymerase, 12.5 mM of each dNTPs, 1.5 mM MgCl₂, and 0.25 µM specific external primers (upstream: 5'-GGAGACCCT GTAGGAGGACCC-3' and downstream: 5'-TCTATAGTGGGGT CGTATTCGTCC-3'; located at nucleotide positions 1625–1645 and 1779–1756, respectively). The PCR comprised an initial denaturation step at 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 58°C for one minute, and extension at 72°C for one minute, with the final extension at 72°C for 10 minutes. Aliquots of the first PCR products (5 µl) were used for nested reactions. In this case, the specific internal primers were added (upstream: 5'-GCAGGCCCTGAGGAGCGATG-3' and downstream: 5'-AAAATGGTTCTGGATCA GCTGGATG-3'; located at nucleotide position 1652–1672 and 1753–1729, respectively). The amplification programme included 25 cycles with annealing at 60°C. The other components of the reaction mixture and parameters of the thermal profile were the same as above. PCR products were analysed electrophoretically on a 2% agarose gel, stained with ethidium bromide.

The nucleotide sequences of the H-ras-1 gene amplified samples were determined by the modified dideoxy chain termination method using the SequiTerm EXCEL™ DNA sequencing kit (Epicentre Technologies, Madison, Wisconsin, USA) for all 35 tumour samples. Templates were sequenced directly

(25 cycles), according to the manufacturer's instructions. The specific sequencing primer H 12/13 (5'-AATGGTTCTGGATCAGCTG-3'; located at nucleotide positions 1751–1733, with an annealing temperature of 60°C) allowed us to establish the primary structure of analysed products in the reverse direction. It prevented the compression effect during sequencing of CG rich fragments. The reaction products were labelled internally by ³²P (γ-³²P-dCTP; 7000 Ci/mmol; Amersham, Amersham, Buckinghamshire, UK), and they were visualised by autoradiography after standard electrophoresis on sequencing gels. For detection of point mutations, we screened the autoradiographic patterns for coexisting bands. The intensity of bands was analysed with molecular analyst software using the Gel Doc 1000 Bio-Rad system. Signals were considered to represent a mutation only if the intensity of the ratio of the new to normal band was > 0.25.

In addition, the possible point mutations in codons 12/13 of H-ras-1 gene were detected in all 35 malignant fibrous histiocytoma specimens by the specific PCR-RFLP method, based on the procedure published previously.²⁶ Briefly, a 102 bp long product of the nested PCR was digested with the NaeI restriction nuclease at 37°C for three hours and the digestion product was electrophoresed using a 2% agarose gel. Only samples with the wild-type sequence within the investigated codons were cleaved, to produce two fragments, 48 and 54 bp long.

Results

All 35 tumours were diagnosed as malignant fibrous histiocytomas and subclassified as storiform (n = 15), pleomorphic (n = 10), or myxoid (n = 10) according to the predominate pattern. We are aware of the great heterogeneity of malignant fibrous histiocytomas, the diagnostic criteria of which are still discussed,²⁷ and our pathologists were very cautious with their classification. The analysed tumours expressed CD 68 and vimentin. There was very little expression of desmin, endothelium, S-100 protein, and CD34. Twenty one cases were classified as high grade sarcomas (G3), whereas 14 were considered to be low grade tumours (G1 or G2).

Codon 12 was not mutated and there were no other changes of the H-ras-1 exon 1 in any of the malignant fibrous histiocytoma specimens. In addition, no tumours diagnosed as the myxoid subtype (10 of 35 tumours) had mutations of codon 12 of the H-ras-1 gene, whereas Bohle *et al* found the typical transversion of G→T in codon 12 as the most characteristic change (with a frequency of ~ 45%) in the myxoid subtype of malignant fibrous histiocytoma.²³ Nine of 32 malignant fibrous histiocytoma cases were described as myxoid subtype in their report, and the overall number of cases investigated and the proportion of myxoid malignant fibrous histiocytomas were exactly the same as in our studies.

Our results indicated that the second hot spot within the H-ras-1 gene, codon 13, and its flanking regions were also normal. Figure 1

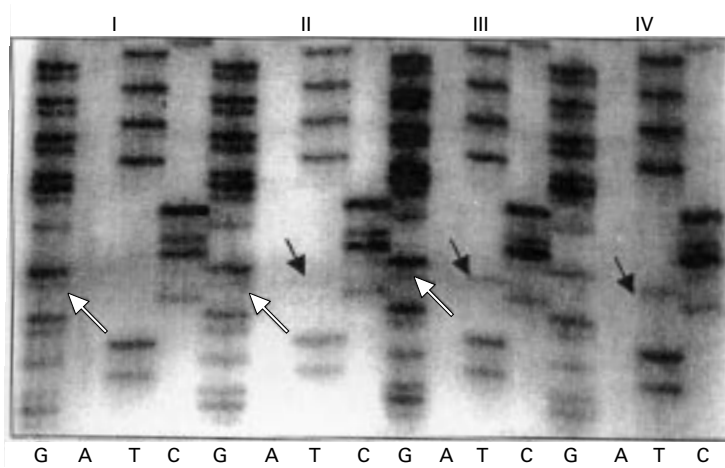
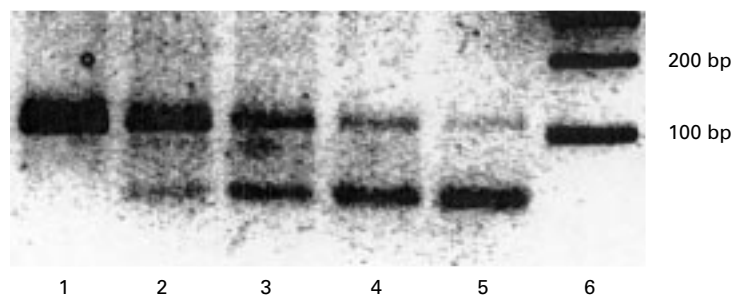


Figure 1 Cycle sequencing data for exon 1 of the *H-ras-1* oncogene. I, template from normal, unmutated DNA; IV, template from T24 cell line DNA, containing homozygously mutated codon 12 of the *H-ras* gene (GGC→GTC); II and III, templates were prepared by mixing normal and mutated DNA (12.5% and 25% DNA from the T24 cell line, respectively). Open headed arrows indicate bands of G nucleotide present in codon 12 of the normal template, which in mutated DNA is transversed to T nucleotide (indicated by a closed arrow).

A



B

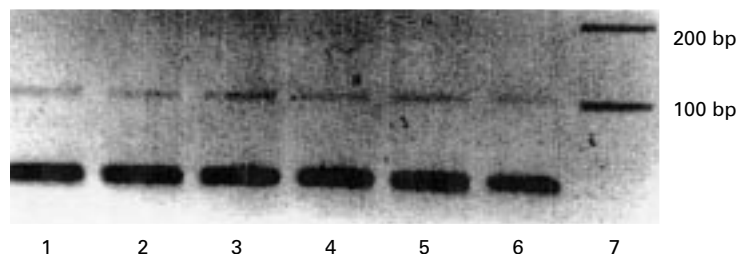


Figure 2 PCR-RFLP analysis of exon 1 of *H-ras-1* oncogene. PCR products were digested with the *NaeI* restriction nuclease, which recognises and cleaves the wild-type sequence only. (A) Lane 1, amplification product of template from T24 cell line DNA; no digestion was seen, only the initial PCR product was visible (102 bp). Lane 5, amplification product of template from non-tumour cells; after DNA cutting, there were two bands corresponding to digestion products (48 and 54 bp) and a trace of initial material (partial digest). Lanes 2, 3, and 4, materials for digestion were prepared by mixing normal and mutated DNA; they contain 85%, 50%, and 25% DNA with transversed G→T from the T24 cell line, respectively. Lane 6, DNA molecular weight standards (100 bp ladders). (B) PCR-RFLP patterns after digestion of selected amplification products of templates isolated from malignant fibrous histiocytoma specimens (lanes 1–6). Lane 7, DNA molecular weight standard (100 bp ladder). In all cases only cleaved fragments were visible.

shows sequencing data generated from the templates containing the region of *H-ras-1* examined, using isotope detection.

Our experiments with mixed templates suggest that in the case of heterozygous mutated

DNA the direct sequencing test allowed the detection of abnormal nucleotides in tumour samples containing 50% transformed cells.

The technique of direct sequencing of PCR products has the advantage of giving detailed information about the codons of interest and their surrounding sequences. When mutational events are limited to known codons (mainly codons 12 or 13 in the *ras* gene) RFLP analysis of PCR products is faster, less expensive, and more sensitive. The results of PCR-RFLP analysis performed using the series of reciprocally diluted normal and mutated DNA amplification products is shown in fig 2A.

It should be noted that this test was two to three times more sensitive than the direct sequencing technique for the detection of mutated template (we should be able to detect a codon 12 mutation in an admixture of tumour cells and normal cells containing 12% tumour cells). Nevertheless, this test did not detect mutations within exon 1 of *H-ras-1* in any examined malignant fibrous histiocytoma samples (fig 2B).

Discussion

Models of neoplastic transformation assume that the accumulation of abnormalities of genes whose protein products are involved in cell cycle regulation (such as p53 and MDM2 genes), or play an important role in signal transduction into the cell (such as *ras* genes), are mainly responsible for uncontrolled tumour growth.²⁸ Reports showing that impaired function of the p53 protein is often accompanied by specific mutation in *ras* family genes support this presumption.²⁹ Because the mutations in *ras* genes are widely recognised in many types of tumours, the search for their presence in sarcoma cells was justified.

Thirty five tumour specimens were reviewed diagnostically before molecular analysis. As described, all selected tumour samples were unequivocally diagnosed as malignant fibrous histiocytomas, excluding other soft tissue sarcomas with a similar phenotype. In addition, their subclassification was performed according to all contemporary criteria. Therefore, we can expect that the tissues used in our study and that of Bohle *et al*²³ with special attention to the nested PCR, to avoid the amplification of *ras* pseudogenes. This PCR-RFLP technique was more sensitive and we introduced it to confirm data from sequencing tests. All control experiments, especially those with the dilution series of DNA from the homozygously mutated cell line, T24, and normal donor unmutated DNA, indicated that we were able to detect the presence of 10–15% of changed template. In such a system, heterozygously mutated tumour cells would have to constitute 20–30% of the analysed sarcoma samples (for homozygously mutated tumour cells, the fraction ~10–15% is sufficient for detection). Our previous immunohistochemi-

cal and molecular evaluations of p53 and MDM2 disturbances (data not shown) revealed that the extent of contamination with normal tissue of our tumour specimens was always low. From our results using both techniques (direct cycle sequencing and PCR-RPLF) to characterise our group of malignant fibrous histiocytoma tumours, it can be concluded that codon 12 mutations of H-ras-1 are not as frequent in this disease as has been suggested²³; we could not identify mutations in this exon in any of our samples. Codon 12 H-ras-1 mutation, which has been described in malignant fibrous histiocytomas previously, is a typical transversion of G→T, which suggests the involvement of unknown external carcinogens in its formation. These types of mutation probably depend on environmental conditions. Alternatively, the differences in results could be connected with the ethnic and genetic heterogeneity of the study population. Evidence supporting the above assumption can be found in an earlier paper,²² which describes another codon 12 H-ras-1 mutation (GGC→GAC) in soft tissue sarcomas, and evidence also comes from other studies concerning different types of cancers.³⁰⁻³¹ Other explanations might be associated with the observation that ras gene abnormalities occur as a late event in the process of tumorigenesis; it may be that our malignant fibrous histiocytoma samples were not at a late enough stage in the transformation process. In addition, Bohle *et al* found that the mutated codon 12 of H-ras-1 exhibited homozygosity in 70% of tumours.²³ There are only a few reports describing homozygous abnormalities of so called "strong" oncogenes and it is difficult to find a clear explanation for this observation. Our contrasting observations cast doubt on the role of H-ras-1 mutation in the carcinogenesis of malignant fibrous histiocytoma and its subtypes (especially the high frequency of H-ras-1 mutation in the myxoid subtype). However, it should be kept in mind that although malignant fibrous histiocytoma is the most common soft tissue sarcoma, very few studies of ras gene mutation have been performed. Both Bos¹¹ and Gill and colleagues²¹ could not find any mutations in the malignant fibrous histiocytoma samples that they studied. These results agree with our observations. Only Wilke *et al* detected codon 12 point mutations (in one third of malignant fibrous histiocytomas tested).²²

In summary, although ras gene mutations are found frequently in several human tumours, their involvement in the oncogenesis of sarcomas is not well defined. The presence of H-ras-1 gene mutations and their real involvement in tumour progression is probably closely connected with the general molecular background of particular sarcomas, such as their p53 and MDM2 gene status.

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