INK4a-ARF alterations in liver cell adenoma

A Tannapfel, C Busse, F Geißler, H Witzigmann, J Hauss, C Wittekind

Background: The INK4a-ARF (CDKN2A) locus on chromosome 9p21 encodes two tumour suppressor proteins, p16INK4a and p14ARF, whose functions are inactivated in many human cancers.

Aims: To evaluate p14ARF and p16INK4a alterations in liver cell adenoma.

Methods: After microdissection, DNA from 25 liver cell adenomas and corresponding normal liver tissue were analysed for INK4a-ARF inactivation by DNA sequence analysis, methylation specific polymerase chain reaction, restriction enzyme related polymerase chain reaction (RE-PCR), mRNA expression, microsatellite analysis, and immunohistochemistry. In addition, microdeletion of p14ARF and p16INK4a were assessed by differential PCR.

Results: Methylation of p14ARF was found in 3/25 cases (12%) and alterations in p16INK4a occurred in 6/25 liver cell adenomas (24%) which correlated with loss of mRNA transcription. We failed to detect microdeletions or specific mutations of both exons. p16INK4a methylation appeared in the context of an unmethylated p14ARF promoter in six cases. In normal liver tissue, p14ARF or p16INK4a alterations were not observed.

Conclusions: Our data suggest that p14ARF methylation occurs independently of p16INK4a alterations in liver cell adenomas. Furthermore, methylation of p14ARF and p16INK4a may be a result of cell cycle deregulation and does not seem to be a prerequisite of malignancy.
a microcapillary tube. Tissue samples were placed in Eppendorf tubes and incubated with proteinase K at 37°C overnight. Proteinase K activity was inactivated by heating to 95°C for 10 minutes. For DNA extraction, standard methods were used: after incubation with proteinase K at 37°C overnight, the tissue was extracted twice in phenol and twice in chloroform, followed by ethanol precipitation.

**Methylation status of the INK4a-ARF locus**

The CpG WIZ p16 methylation assay kit was used (OncoImmuno, Gaithersburg, Maryland, USA) according to the manufacturer’s instructions. After an initial bisulphide reaction to modify the DNA, polymerase chain reaction (PCR) amplification with specific primers was performed to distinguish methylation from unmethylated DNA. Primers specific for unmethylated p16 (5'-TATTAGAGGGGTGGTGATTG-3', 5'-CAACCCCAACCAACACATA-3) or methylated p16 (5'-TATTAGAGGGTGGTGGTGATTG-3', 5'-GACCCCGAAACCGCGACGTA-3) were used. DNA (7 μg/100 μl) was denatured by 0.2 M NaOH for 10 minutes at room temperature. DNA Modification Reagent I was added, incubated for 24 hours at 50°C, and subsequently purified by DNA Modification Reagents II and III.

Bisulphide converted DNA from corresponding normal liver tissue from each patient served as a negative control, as Bisulphide converted DNA from corresponding normal liver tissue was directly electrophoresed on a 3% agarose gel, stained (1.25 nM), and visualised under UV illumination. The reaction was then performed for 20 minutes followed by ethanol precipitation. For hot start PCR, the DNA was completed with 0.3 M NaOH treatment for five minutes followed by ethanol precipitation for hot start PCR. The PCR mixture contained Universal PCR Buffers (1×), 4 dNTPs (1.25 mM), and U or M primers (300 ng each per reaction). Annealing temperature was 65°C for 30 cycles. The PCR product was directly electrophoresed on a 8% polyacrylamide gel, stained, and visualised.

**Multiplex RT-PCR**

To compare relative levels of p16mRNA and p14mRNA, multiplex reverse transcription-PCR (RT-PCR) was performed. Total RNA was extracted from 30 μg of microdissected LCA tissue by TRizol reagents (Gibco BRL, Rockville, Maryland, USA). After ethanol washing and drying, RNA was suspended in 60 μl of diethyl pyrocarbonate treated water. After concentration determination, 2 μg of total RNA were subjected to a reverse transcription reaction using random oligonucleotide primers and superscript II reverse transcriptase (Gibco BRL) in a 20 μl reaction volume for 60 minutes at 42°C. The RT reaction product (1 μl) was then amplified by PCR using the forward primer for exons 1α and 1β and the reverse primer for exon 2 of the p16mRNA-p14mRNA. The primers were as follows: forward exon 1α (sense 1): 5'-GCTGCCCAACCAACACATA-3; exon 1β (sense 2): 5'-GCCCTGCTGCTGATGCTACTGA-3; and reverse primer (antisense) 5'-ACACCAGGCTGTCACGAAAGT. Hot start PCR was performed for 35 cycles (95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 60 seconds). The sizes of the products were 179 bp for p16mRNA and 200 bp for p14mRNA, respectively. PCR products were electrophoresed on a 2% agarose gel and stained. β-actin amplification was performed to show RNA quality.

**Allelic dosage analysis of gene loss**

Allergic dosage analysis of the p14mRNA genes was performed using differential PCR. DNA fragments were amplified in exon 1β of p14mRNA, exon 3 of p16mRNA, and exon 2 using the following primers: p14arf exon 1b: ARF2F 5'-CCCTCGTGCTGATGCTACTAGA-3 and ARF2R 5'-AGAGCACAAACAGAGGATGAA-3, and p16mRNA exon 3: p16ex3F 5'-GCAAGTAAAGAAGACAGAGAG-3 and p16ex3R 5'-ATGAGACATTSTGTTGAG-3. After PCR, the amplified products were electrophoresed on a 2% agarose gel and stained. The PCR products were visualised under UV illumination.

**Allelic dosage analysis of loss of heterozygosity and homozygous deletion, and DNA sequencing for the INK4a-ARF (CDKN2A) locus**

Allelic dosage analysis of the p14mRNA and p16mRNA genes was performed using differential PCR. DNA fragments were amplified in exon 1β of p14mRNA, exon 3 of p16mRNA, and exon 2 using the following primers: p14arf exon 1b: ARF2F 5'-CCCTCGTGCTGATGCTACTAGA-3 and ARF2R 5'-AGAGCACAAACAGAGGATGAA-3, and p16mRNA exon 3: p16ex3F 5'-GCAAGTAAAGAAGACAGAGAG-3 and p16ex3R 5'-ATGAGACATTSTGTTGAG-3. After PCR, the amplified products were electrophoresed on a 2% agarose gel and stained. β-actin amplification was performed to show RNA quality.

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Figure 1  Analysis of p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} in three liver cell adenomas (case Nos 1, 10, and 11; same patients as in table 1). (A) p14\textsuperscript{ARF} analysis with restriction enzyme related-polymerase chain reaction (RE-PCR). The methyl sensitive restriction enzymes used for RE-PCR are indicated (Hpa\textsuperscript{II}, Ksp\textsuperscript{I}); digestion with the non-methyl sensitive enzyme Msp\textsuperscript{I} serves as a negative control and undigested DNA (control) serves as a positive control. The p14\textsuperscript{ARF} gene is methylated in case No 11 and unmethylated in case Nos 1 and 10. (B) p16\textsuperscript{INK4a} analysis with RE-PCR. Similar to (A), the methyl sensitive restriction enzymes used for RE-PCR are indicated (Hpa\textsuperscript{II}, Ksp\textsuperscript{I}); digestion with the non-methyl sensitive enzyme Msp\textsuperscript{I} serves as a negative control and undigested DNA (control) serves as a positive control. Methylation of p16\textsuperscript{INK4a} is detected in case No 1, but not in case Nos 10 and 11. (C) p16\textsuperscript{INK4a} analysis using methylation specific polymerase chain reaction (MSP). Bisulphite treated DNA (which changes the unmethylated but not the methylated cytosines into uracil) is subjected to PCR amplification using primers designed to anneal specifically to the methylated bisulphite modified DNA. MSP results are expressed as unmethylated p16 specific bands (U) or methylated p16 specific bands (M). Bisulphite converted DNA from normal corresponding liver tissue (N) served as a negative control, as indicated by the presence of the U but not the M band. Similar to (B), methylation of p16\textsuperscript{INK4a} was detected in case No 1 but not in case Nos 10 and 11. (D) Results of multiplex reverse transcription-PCR (RT-PCR) of p14 mRNA (upper line corresponding to 200 bp) and p16 mRNA (lower line corresponding to 179 bp) for case Nos 1, 10, and 11. (E) Immunostaining of p16\textsuperscript{INK4a} protein in liver cell adenoma (LCA). Case No 1 shows methylated p16\textsuperscript{INK4a} and complete loss of p16\textsuperscript{INK4a} (LCA cells negative for p16 protein) (original magnification x10). p16\textsuperscript{INK4a} is detectable in case Nos 10 and 11 (dark reaction product within the cell nuclei) (original magnification x20 and x40). (F) Immunostaining of p14\textsuperscript{ARF} protein in LCA. Case No 1 shows unmethylated p14\textsuperscript{ARF} and strong immunoreactivity of the tumour cells for p14 protein (dark reaction product within the tumour cell nuclei). The tumour surrounding fibrous capsule (arrows) is negative (original magnification x5). Case No 11 shows a methylated p14\textsuperscript{ARF} and complete protein loss within the tumour tissue (original magnification x20).
non-denaturing environment. Coding sequences and flanking intronic sequences of exons 1α, β, and 2 of the INK4a-ARF gene were analysed by PCR-SSCP. Primer sequences for exons 1α, β, and 2 have been described previously.16 Exon 1β was analysed through two overlapping PCR products generated with the primer pairs P14F1 (5′ TCAGGGAAAGGGCGGTGCCG 3′) and P14R1 (5′ GCCGGGGGATGTGAACCA 3′), which generated a 245 bp product, and the primer pair P14F2 (5′ GCCGGGAGGTGAACCA 3′), which generated a 245 bp product, and the primer pair P14F2 (5′ GCCGGGAGGTGAACCA 3′), which generated a 245 bp product. The primers were labelled with 32P-ATP and each sample was subjected to PCR analysis (denaturing for 30 seconds, annealing for 45 seconds, extension for 30 seconds at 72°C, respectively). The PCR products were electrophoresed, and the gels dried and autoradiographed. Variant SSCP bands were cut out from the gel and the DNA eluted. Variant bands and 3′ SSCP bands were cut out from the gel and the DNA eluted.

Immunohistochemical analysis and assessment
Immunohistochemical analysis was performed as described previously.16 In all cases tumour and non-neoplastic liver tissue was examined.

The following antibodies were used: p16 (polyclonal; rabbit, dilution 1:500; Pharmingen, San Diego, California, USA), and p14 (polyclonal; rabbit, dilution 1:100; Zymed Laboratories, South San Francisco, California, USA).

Sections known to stain positively were included in each batch and negative controls were also performed by replacing the primary antibody with mouse or goat ascites fluid (Sigma-Aldrich Biochemicals, St Louis, Missouri, USA).

RESULTS
Analysis of INK4a-ARF deletions and mutations
Twenty five normal/tumour pairs were interpreted for allelic dosage analysis (table 1, fig 2). The allelic balance of the two genes was determined using the interferon γ gene as an internal control (fig 2). The two genes, p14ARF and p16ARF, were expressed in all cases examined; deletions were not observed. No exclusive loss of either p14ARF or p16ARF was found in our tumours. Loss of heterozygosity analysis revealed an identical status of the microsatellite markers used in paired samples of LCA and corresponding liver (fig 3).

Mutations of exons 1 and 2 were analysed by SSCP-PCR followed by direct sequencing of the cases with anomalous migrating bands. In nine cases, abnormal bands were visible. However, we failed to detect specific mutations within both exons. In one case, a polymorphism was identified in normal liver but not within LCA tissue (c442G >A; A148T).

Methylation status of the p14ARF and p16ARF genes
Promoter methylation of p14ARF was present in 3/25 cases (12%). In all patients, corresponding non-neoplastic liver tissue was also analysed; no p14ARF promoter methylation was observed in any case. Analysis of the methylation status of the adjacent p16ARF gene revealed that 6/25 LCA (24%) examined showed aberrant methylation at the 5′ CpG island. Despite microdissection, amplification of unmethylated templates was also detected to some degree, probably because of contaminated normal intratumorous tissue (fibroblasts, endothelial cells, inflammatory cells). In normal LCA surrounding liver tissue, methylation of p14ARF or p16ARF was not observed.

All six LCA with methylated p16ARF exhibited an unmethylated p14ARF promoter. A coincidence of both p14ARF and p16ARF methylation was not found. Thus the methylation status of p14ARF and p16ARF promoters does not seem to be directly related.

Real time PCR of those samples with a methylated p16ARF gene showed a level of methylation of approximately 75%.

All six cases with aberrant methylation of the p16ARF or p14ARF gene showed complete loss of immunoreactivity (fig 1E, F) within the tumour tissue. In the 19 cases shown to lack p16ARF promoter methylation, nuclear staining of p16ARF protein was observed in nearly all LCA cells with a moderate to
downregulated p16 detected in 22/25 tumours (fig 1D). Among the tumours with agus, and pancreas.

In early preneoplastic lesions in the lung, stomach, oesoph-

corresponding promoters was observed in six and three cases,

methylated p14 INK4a-ARF locus in LCA.

aberrant methylation is a major mechanism of inactivation of the INK4a-ARF locus in LCA.

DISCUSSION

Recently, aberrant methylation of the p16INK4a promoter has been reported not only in various types of carcinomas but also in early preneoplastic lesions in the lung, stomach, oesoph-

Our study showed that the p14ARF promoter was inactivated in 12% of cases. In 24% of all LCA examined, promoter methylation of the neighbouring gene, p16INK4a, was observed. We failed to detect simultaneous methylation of both genes and conclude that p14ARF methylation is independent of p16INK4a. Thus the p14ARF promoter demonstrates selective epigenetic silencing independent of that of p16INK4a. The strong correlation between promoter methylation and transcriptional inac-

In concordance with data reported for cell lines, we failed to detect specific mutations of the p14ARF gene. p14ARF can also be lost by (homozygous) deletion but this loss also targets p16INK4a in the vast majority of cases. Only a few examples currently exist of specific p14ARF deletions that spare the remainder p16INK4a coding region: a melanoma cell line and a glioma xenograft.21

In human cells, transcriptional silencing usually involves methylation of CpG rich sequences (CpG islands) in the promoters of affected genes. Such silencing is clonal and thought to be physiologically irreversible in somatic cells. Neoplastic cells often display aberrant methylation of multiple genes, including genes that regulate critical processes such as cell cycle control, DNA repair, and angiogenesis.22 23 24 The cause(s) of aberrant promoter methylation in neoplastic cells remains to be elucidated. It has been proposed that age related methylation identifies and contributes to an acquired predis-

Table 1 Pathohistological data and INK4a-ARF alterations

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RE-PCR, restriction enzyme related-polymerase chain reaction; MSP, methylation specific PCR; EXP, gene mRNA expression analysed by reverse transcription PCR; ND, not detected (wild-type, both alleles expressed as defined by multiplex PCR); NI, not informative.
REFERENCES


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