Liver cell adenoma (LCA) is the most important benign epithelial tumour of the liver, with an incidence of approximately 3/1 000 000 new cases per year. LCA are pathogenetically related to the use of oral contraceptives, androgenic steroid therapy, and have also been reported in association with glycogen storage disease. Microscopically, the neoplasia is composed of well differentiated uniform cords of proliferating hepatocytes. Normal portal tracts are absent, tumour cells are uniform in size and shape but atypical pleomorphic cells with distorted hyperchromatic nuclei may be seen. Transformation of LCA to hepatocellular carcinoma has been described but is extremely rare. To date, the cellular and molecular mechanisms leading to uncontrolled proliferation of hepatocytes remain unclear. Great insights will come from integrating the signals of different pathways operating at cell cycle regulation, cellular proliferation, and apoptosis. There is evidence that alterations in the INK4a-ARF locus, which maps to chromosome 9p21, may contribute to the development of liver tumours. The INK4a-ARF or CDKN2A locus codes for two different proteins, p16\(^{INKA}\) and p14\(^{ARF}\), whose functions are inactivated in many human cancers.

## Background

The INK4a-ARF (CDKN2A) locus on chromosome 9p21 encodes two tumour suppressor proteins, p16\(^{INKA}\) and p14\(^{ARF}\), whose functions are inactivated in many human cancers.

## Aims

To evaluate p14\(^{ARF}\) and p16\(^{INKA}\) alterations in liver cell adenoma.

## Methods

After microdissection, DNA from 25 liver cell adenomas and corresponding normal liver tissue were analysed for INK4-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 p14\(^{ARF}\) and p16\(^{INKA}\) were assessed by differential PCR.

## Results

Methylation of p14\(^{ARF}\) was found in 3/25 cases (12%) and alterations in p16\(^{INKA}\) 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. p16\(^{INKA}\) methylation appeared in the context of an unmethylated p14\(^{ARF}\) promoter in six cases. In normal liver tissue, p14\(^{ARF}\) or p16\(^{INKA}\) alterations were not observed.

## Conclusions

Our data suggest that p14\(^{ARF}\) methylation occurs independently of p16\(^{INKA}\) alterations in liver cell adenomas. Furthermore, methylation of p14\(^{ARF}\) and p16\(^{INKA}\) 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 (Oncor Inc, 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 methylated from unmethylated DNA. Primers specific for unmethylated p16 (5′-TTATAGAGGGTTGGTGATTP-3, 5′-CAACCCCAAAACA CAACCATAA-3) or methylated p16 (5′-TTATAGAGGGTG GGGCGGTATCGC-3, 5′-GACCCCCAGCCGGACCG TAA-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 in diethyl pyrocarbonate treated water. 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 3% agarose gel, stained with ethidium bromide, and visualised under UV illumination.

**Multiplex RT-PCR**

To compare relative levels of p16\textsuperscript{iso} and p14\textsuperscript{iso} mRNA, multiplex reverse transversion-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 primers of exons 1α and 1β and the reverse primer for exon 2 of the p16\textsuperscript{iso}-p14\textsuperscript{iso} gene. The primers were as follows: forward exon 1α (sense 1): 5′-GCTGCGCAGCAGCGCATTA-3; exon 1β (sense 2): 5′-CTCTGGTGTAGTCTGATGA-3; and reverse primer (antisense) 5′-ACCCATGTCGCTGCTGCTGAGA-3. 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 p16\textsuperscript{iso} and 200 bp for p14\textsuperscript{iso}, 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 loss of heterozygosity and homozygous deletion, and DNA sequencing for the INK4a-ARF (CDKN2A) locus**

Allelic dosage analysis of the p14\textsuperscript{iso} and p16\textsuperscript{iso} genes was performed using differential PCR. DNA fragment sizes were amplified in exon 1β of p14\textsuperscript{iso}, exon 3 of p16\textsuperscript{iso}, and exon 2 using the following primers: p14arf exon 1β: ARF2F 5′-GCTGCCCACGCACCGAATA-3; exon 1β 5′-GGGCGGATCGC-3; 5′-GACCCCGAACCGCGACCG TA-3; and U probe: 5′-GGGCGGAGAT; reverse: GCGGCTGCTGCCCTAGA. For hot start PCR, the PCR product was amplified using the following probes and primers: E5: 5′-CTCTGGTGTAGTCTGATGA-3; and reverse primer (antisense) 5′-ACCCATGTCGCTGCTGCTGAGA-3. 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 p16\textsuperscript{iso} and 200 bp for p14\textsuperscript{iso}, respectively. PCR products were electrophoresed on a 2% agarose gel and stained. β-actin amplification was performed to show RNA quality.
Figure 1  Analysis of p14<sup>ARF</sup> and p16<sup>INK4a</sup> in three liver cell adenomas (case Nos 1, 10, and 11; same patients as in table 1). (A) p14<sup>ARF</sup> analysis with restriction enzyme related-polymerase chain reaction (RE-PCR). The methyl sensitive restriction enzymes used for RE-PCR are indicated (HpaII, KspI); digestion with the non-methyl sensitive enzyme MspI serves as a negative control and undigested DNA (control) serves as a positive control. The p14<sup>ARF</sup> gene is methylated in case No 11 and unmethylated in case Nos 1 and 10. (B) p16<sup>INK4a</sup> analysis with RE-PCR. Similar to (A), the methyl sensitive restriction enzymes used for RE-PCR are indicated (HpaII, KspI); digestion with the non-methyl sensitive enzyme MspI serves as a negative control and undigested DNA (control) serves as a positive control. Methylation of p16<sup>INK4a</sup> is detected in case No 1, but not in case Nos 10 and 11. (C) p16<sup>INK4a</sup> 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<sup>INK4a</sup> 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<sup>INK4a</sup> protein in liver cell adenoma (LCA). Case No 1 shows methylated p16<sup>INK4a</sup> and complete loss of p16<sup>INK4a</sup> (LCA cells negative for p16 protein) (original magnification ×10). p16<sup>INK4a</sup> is detectable in case Nos 10 and case 11 (dark reaction product within the cell nuclei) (original magnification ×20 and ×40). (F) Immunostaining of p14<sup>ARF</sup> protein in LCA. Case No 1 shows unmethylated p14<sup>ARF</sup> 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 ×5). Case No 11 shows a methylated p14<sup>ARF</sup> and complete protein loss within the tumour tissue (original magnification ×20).
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. Exon 1β was analysed through two overlapping PCR products generated with the primer pairs P14F1 (5′ TCAGGGAAAGGCGCTGC 3′) and P14R1 (5′ GCCCGGGATGTAACAC 3′), which generated a 245 bp product, and the primer pair P14F2 (5′ GCCCGGAGTGAGGGTTT 3′) and P14R2 (5′ CACCGCGGTATCTCCTC 3′), which generated a 257 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 94°C, 55–60°C, and 72°C, respectively). The PCR products were electrophoresed, and the gels dried and autoradiographed. Variant bands were cut out from the gel and the DNA eluted. Variant bands and 3 μl of the eluted DNA were used as templates for unlabelled PCR. After purification of the PCR products, sequencing analysis was performed using the DNA Sequense Kit (Amersham, Germany) and an automatic sequencing analyser (ABI 373; Applied Biosystems-Perkin-Elmer, Germany). All mutations found were confirmed by direct sequencing of the amplified tumour and corresponding non-tumorous tissue.

Immunohistochemical analysis and assessment

Immunohistochemical analysis was performed as described previously. 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, p14 and p16, were expressed in all cases examined; deletions were not observed. No exclusive loss of either p14 or p16 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 intratumorous tissue (fibroblasts, endothelial cells, inflammatory cells). In normal LCA surrounding liver tissue, methylation of p14 or p16 was not observed.

Methylation status of the p14 and p16 genes

Promoter methylation of p14 was present in 3/25 cases (12%). In all patients, corresponding non-neoplastic liver tissue was also analysed; no p14 promoter methylation was observed in any case. Analysis of the methylation status of the adjacent p16 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 p14 or p16 was not observed.

All six LCA with methylated p16 exhibited an unmethylated p14 promoter. A coincidence of both p14 and p16 methylation was not found. Thus the methylation status of p14 and p16 promoters does not seem to be directly related.

Real time PCR of those samples with a methylated p16 gene showed a level of methylation of approximately 75%.

All six cases with aberrant methylation of the p16 or p14 gene showed complete loss of immunoreactivity (fig 1E, F) within the tumour tissue. In the 19 cases shown to lack p16 promoter methylation, nuclear staining of p16 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-
been reported not only in various types of carcinomas but also
in 12% of cases. In 24% of all LCA examined, promoter meth-
tion between promoter methylation and transcriptional inac-
conclude that p14 alterations p16 alterations
methylation is independent of p16 
 alterations in liver cell adenoma.

DISCUSSION
Recently, aberrant methylation of the p16 promoter has been
even in early preneoplastic lesions in the lung, stomach, oesoph-
agous, and pancreas.17–21 Ours is the first study to examine
alterations in the INK4a-ARF (also termed CDKN2A) locus
on chromosome 9p21 in LCA, the most important benign
epithelial tumour of the liver. We examined the status of
INK4a-ARF alterations in liver cell adenoma.

Table 1  Pathohistological data and INK4a-ARF alterations

<table>
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In concordance with data reported for cell lines, we failed to
detect specific mutations of the p14 or p16 gene.24 p14 can also be lost by (homozygous) deletion but this loss also
targets p16 in the vast majority of cases.18 Only a few examples currently exist of specific p14 deletions that spare
the remainder p16 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.12,15,24 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-
position to neoplasia (for example, colon cancer) because it parallels an age related increased cancer incidence and has the
potential to alter the physiology of aging cells and tissues.25–27 This hypothesis predicts that higher levels of age related
methylation may be present in conditions of rapid cell turnover
that mimic premature aging. In LCA, an increase in cellular
proliferation is often visible histologically. The proliferative
activity of the neoplastic hepatocytes is significantly higher
than in adenoma surrounding non-neoplastic liver tissue.2,17 Therefore, we hypothesise that methylation and consecutive
silencing of the p16 and p14 promoter may cause induction
of increased cell turnover via affecting the G1/S phase
transition of the cell cycle. In contrast with Rashid et al who
found aberrant methylation of p16 in approximately 73%
of tubulovillous colon adenoma,28 a clear precancerous lesion,
we detected aberrant methylation only in 24% of LCA.
Together with the observation that altered methylation is also
observed in liver cirrhosis,29 our data favour the hypothesis that
methylation is a phenomenon of increased cellular proliferation
and immortalisation rather than a conditio-sine-qua-

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