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, 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 INK4a-ARF inactivation by DNA sequence analysis, methylisation 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.

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 neoplasm 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\(^{ARF}\), both involved in cell cycle regulation. These two proteins are characterised by two distinct promoters and first exons spliced to a common exon 2 in different reading frames; exons 1α, 2, and 3 for p16\(^{INKa}\) and exons 1β, 2, and 3 for p14\(^{ARF}\). The tumour suppressor gene p16\(^{INKa}\) is believed to encode a negative regulatory protein that controls the progression of eucaryotic cells through the G1 phase of the cell cycle by interacting with CDK4 and inhibiting its kinase activity. In the absence of functional p16 protein, CDK4 binds to cyclin D and phosphorylates pRb which stimulates entry into the S phase. The p16\(^{INKa}\) gene is inactivated by mutations, homozygous deletions, or gene methylation in many tumours of diverse origin. p14\(^{ARF}\), generated through an alternative splicing process that replaces the first exon, has been shown to function as a growth suppressor. p14\(^{ARF}\) specifically activates the p53 pathway. p14\(^{ARF}\) stabilises p53 by inhibiting MDMD2 dependent p53 degradation, thereby inducing cell cycle arrest or apoptosis, depending on the stimulus. Data have shown that p14\(^{ARF}\) binds to MDMD2 through an NH2 terminal domain encoded by exon 1 whereas a functional domain is encoded by exon 2. Activation of p14\(^{ARF}\) (in response to an oncogenic signal such as c-myc, activated ras) leads to localisation and sequestration of MDMD2 in the nucleolar compartment, thereby stabilising p53 by preventing MDMD2-p53 from undergoing ubiquitin mediated degradation. To date, data concerning INK4a-ARF alterations in benign tumours of the liver are lacking.

To gain insights into the role of the INK4a-ARF locus in the development of LCA, mutational and expression analyses of p16\(^{INKa}\) and p14\(^{ARF}\) were performed in a large group of patients with this disease.

MATERIALS AND METHODS

Patients and tissue samples
Twenty five patients with LCA undergoing partial hepatectomy (segmental or lobar resection) between 1990 and 1999 were included in this retrospective study. Each tumour was re-evaluated with regard to typing (WHO 2000). In all cases, slides prepared from four different paraffin blocks of tissue, sampled from different tumour areas, were examined.

DNA samples
For each LCA sample, the histopathological lesions of interest were first identified on routinely stained slides. Parallel sections were cut with the microtome set at 6 µm, and the slides dried overnight at 37°C. Corresponding areas of interest were delineated and microdissected after rapid staining with haematoxylin and eosin. Thereafter the tissue was scraped off the slide (sections were covered by 25 µl of Tris buffer 0.05 mol) with the tip of a sealed glass pipette and then sucked into

Abbreviations: LCA, liver cell adenoma; MSP, methylation specific polymerase chain reaction; RE-PCR, restriction enzyme related polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SSCP, single strand conformational polymorphism.
a microcapillary tube. Tissue samples were placed in Eppendorf tubes and incubated with protease K at 37°C overnight. Protease K activity was inactivated by heating to 95°C for 10 minutes. For DNA extraction, standard methods were used: after incubation with protease 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 (OncoInc, 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′-TTATAGGAGGGTGGTGTAGTP-3′, 5′-CAACCCCAACAAACACATAA-3′) or methylated p16 (5′-TTATAGGAGGGTGGTGTAGTP-3′, 5′-GCCGGGATCGCC-3′, 5′-GACCCCCGAGCGGACCCTA-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 the presence of 50 µl of water. The bisulphide modification of 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×), 4dNTPs (1.25 µM), 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. Bisulphide converted DNA from corresponding normal liver tissue from each patient served as a negative control, as indicated by the presence of the unmethylated but not the methylated band. To control the efficacy of bisulphide treatment, a primer set was used for unmethylated or wild-type ("w") (5′-CAGAGGGTGGGGCGGACCGA-3′ and 5′-GGGCCGCGCCGGGG; M probe: 5′-FAM-AACCCCGAACCGGCG-TAMRA; and U probe: 5′-FAM-CAACCCACAAAAACACACACATCCAGC-TAMRA). The sensitivity and specificity of real time PCR was determined using cloned fragments of bisulphide modified DNA as a fragment.

Multiplex RT-PCR
To compare relative levels of p16 exon 1 and p14 exon 1, 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 amplifed by PCR using the forward primers of exons 1a and 1b and the reverse primer for exon 2 of the p16 exon-p14 exon gene. The primers were as follows: forward exon 1a (sense 1): 5′-GCTGCCCACCGCCCGATA-3′; exon 1b (sense 2): 5′-CCTCGTGTCGTACTGTA-3′; and reverse primer (antisense) 5′-ACCACCCACGTTGGCGGAAA-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 exon 1a and 200 bp for p14 exon 1b, 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 exon 1b and p16 exon 1b genes was performed using differential PCR. DNA fragments were amplified in exon 1b of p14 exon 1, exon 3 of p16 exon 2, and exon 2 using the following primers: p14arf exon 1b: ARF2F 5′-CCCTCGTGTCGTACTGAG-3′ and ARF2R 5′-AAATGCGTGTGAACCCGAATG-3′; exon 1b: 5′-GCTGCCCACCGCCCGATA-3′; exon 1b (sense 1): 5′-GCTGCCCACCGCCCGATA-3′; exon 1b (sense 2): 5′-CCTCGTGTCGTACTGTA-3′; and reverse primer (antisense) 5′-ACCACCCACGTTGGCGGAAA-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 exon 1b and 200 bp for p14 exon 1b, 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 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′ TCAGGGAAGGGCGGGTGCG 3′) and P14R1 (5′ GCCGCAGGTGAACCA 3′), which generated a 245 bp product, and the primer pair P14F2 (5′ GCCGCAGGTGAAGGTTTT 3′) and P14R2 (5′ CACCGCGGTTATCTTCTC 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 SSCP 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 Sequenase Kit (Amersham, Germany) and an automatic sequencing analyser (ABI 373; Applied Biosystems-Perkin-Elmer, South San Francisco, California, 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 p16INK4A, were expressed in all cases examined; deletions were not observed. No exclusive loss of either p16INK4A or p14ARF 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 p16INK4A 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 p16INK4A 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 p16INK4A was not observed.

All six LCA with methylated p16INK4A exhibited an unmethylated p14ARF promoter. A coincidence of both p14ARF and p16INK4A methylation was not found. Thus the methylation status of p14ARF and p16INK4A promoters does not seem to be directly related.

Real time PCR of those samples with a methylated p16INK4A gene showed a level of methylation of approximately 75%.

All six cases with aberrant methylation of the p16INK4A or p14ARF gene showed complete loss of immunoreactivity (fig 1E, F) within the tumour tissue. In the 19 cases shown to lack p16INK4A promoter methylation, nuclear staining of p16INK4A protein was observed in nearly all LCA cells with a moderate to
strong intensity of immunoreactivity. In normal liver tissue, p16\(^{\text{INK4a}}\) protein was detected in all cases (fig 1E, F). Three LCA with a methylated p14\(^{\text{ARF}}\) promoter lacked specific p14\(^{\text{ARF}}\) immunostaining (fig 1E, F).

**Multiplex RT-PCR for p16\(^{\text{INK4a}}\) and p14\(^{\text{ARF}}\) mRNA**

Using specific sense primers for exon 1 and a common reverse primer for exon 2, both transcripts were simultaneously amplified in a single reaction. p16\(^{\text{INK4a}}\) mRNA was amplified in 19/25 cases and p14\(^{\text{ARF}}\) transcripts were detected in 22/25 tumours (fig 1D). Among the tumours with downregulated p16\(^{\text{INK4a}}\) or p14\(^{\text{ARF}}\) mRNA, methylation of the corresponding promoters was observed in six and three cases, respectively.

**DISCUSSION**

Recently, aberrant methylation of the p16\(^{\text{INK4a}}\) promoter has been reported not only in various types of carcinomas but also in early preneoplastic lesions in the lung, stomach, oesophagus, and pancreas.\(^{25,26}\) 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 p14\(^{\text{ARF}}\) and p16\(^{\text{INK4a}}\) simultaneously to answer the question of whether alterations in these genes may function as cooperative or alternative mechanisms in the pathogenesis of these tumours.

Our study showed that the p14\(^{\text{ARF}}\) promoter was inactivated in 12% of cases. In 24% of all LCA examined, promoter methylation of the neighbouring gene, p16\(^{\text{INK4a}}\), was observed. We failed to detect simultaneous methylation of both genes and conclude that p14\(^{\text{ARF}}\) methylation is independent of p16\(^{\text{INK4a}}\). Thus the p14\(^{\text{ARF}}\) promoter demonstrates selective epigenetic silencing independent of that of p16\(^{\text{INK4a}}\). The strong correlation between promoter methylation and transcriptional inactivation, as examined by multiplex RT-PCR, indicates that aberrant methylation is a major mechanism of inactivation of the INK4a-ARF locus in LCA.

In concordance with data reported for cell lines, we failed to detect specific mutations of the p14\(^{\text{ARF}}\) or p16\(^{\text{INK4a}}\) gene.\(^{27}\) p14\(^{\text{ARF}}\) can also be lost by (homozygous) deletion but this loss also targets p16\(^{\text{INK4a}}\) in the vast majority of cases.\(^{28,29}\) Only a few examples currently exist of specific p14\(^{\text{ARF}}\) deletions that spare the remainder p16\(^{\text{INK4a}}\) coding region: a melanoma cell line and a glioma xenograft.\(^{30}\)

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.\(^{24,31,32}\) 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 predisposition 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.\(^{25,33}\) 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.\(^{34,35}\) Therefore, we hypothesise that methylation and consecutive silencing of the p16\(^{\text{INK4a}}\) and p14\(^{\text{ARF}}\) 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\(^{\text{INK4a}}\) in approximately 73% of tubulovillois colon adenoma,\(^{36}\) a clear precancersous lesion, we detected aberrant methylation only in 24% of LCA. Together with the observation that altered methylation is also observed in liver cirrhosis,\(^{9}\) our data favour the hypothesis that methylation is a phenomenon of increased cellular proliferation and immortalisation rather than a conditio sine qua non of malignant transformation.
REFERENCES


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