INK4α-ARF alterations in liver cell adenoma

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Liver cell adenoma (LCA) is the most important benign epithelial tumour of the liver, with an incidence of approximately 3/1000000 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 INK4α-ARF locus, which maps to chromosome 9p21, may contribute to the development of liver tumours.

The INK4α-ARF or CDKN2A locus codes for two different proteins, p16INK4a and p14ARF, 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 p16INK4a and exons 1β, 2, and 3 for p14ARF. The tumour suppressor gene p16INK4a 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 p16INK4a gene is inactivated by mutations, homozgyous deletions, or gene methylation in many tumours of diverse origin. p14ARF, generated through an alternative splicing process that replaces the first exon, has been shown to function as a growth suppressor. p14ARF specifically activates the p53 pathway, p14ARF stabilises p53 by inhibiting MDM2 dependent p53 degradation, thereby inducing cell cycle arrest or apoptosis, depending on the stimulus. Data have shown that p14ARF binds to MDM2 through an NH2 terminal domain encoded by exon 1 whereas a functional domain is encoded by exon 2. Activation of p14ARF (in response to an oncogenic signal such as c-myc, activated ras) leads to localisation and sequestration of MDM2 in the nucleolar compartment, thereby stabilising p53 by preventing MDM2-p53 from undergoing ubiquitin mediated degradation. To date, data concerning INK4α-ARF alterations in benign tumours of the liver are lacking.

To gain insights into the role of the INK4α-ARF locus in the development of LCA, mutational and expression analyses of p16INK4a and p14ARF 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 a microfuge.

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 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 (OncorInc, 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'-TTATAGAGGGTGAGTGATGF-3', 5'-CAACCCCAACCAACATTAAAA-3') or methylated p16 (5'-TTATAGAGGTTGAGGCGGATCGC-3', 5'-GACCCCCCAACGGGAGCC-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 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. The CpG WIZ p16 methylation assay kit was used (OncorInc, 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'-TTATAGAGGGTGAGTGATGF-3', 5'-CAACCCCAACCAACATTAAAA-3') or methylated p16 (5'-TTATAGAGGTTGAGGCGGATCGC-3', 5'-GACCCCCCAACGGGAGCC-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 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.

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

Allelic dosage analysis of the p14ARF and p16INK4a genes was performed using differential PCR. DNA fragments were amplified in exon 1B of p14ARF, exon 3 of p16INK4a, and exon 2 using the following primers: p14arf exon 1B: ARF2F 5'-CTCGTGCTGATGCTAGAG-3' and ARFR2 5'-AAGTGGTTGAACCCGAAAT-3'; p16 exon 3: p16ex3F: 5'-CGATTGAAAAGACAGAGAG-3' and p16ex3R 5'-ATGACATTACCGTGGTAGTG-3'; interferon γ: INF2F2dF 5'-GAGGGATCTGCAGAAGAGAGAG-3' and INF2RdR 5'-GGAGCAACAAACAGAGATGAA-3'. As a negative control, a glioblastoma cell line (LN343) with a known deletion of the INK4a-ARF locus was used. For positive controls, the hepatocellular carcinoma cell line HepG2 with an intact INK4a-ARF locus was used. The ratio of DNA fragment intensity in HepG2 between exon 1B or exon 3 and the internal control interferon γ was used to normalise the results. Hemizygous deletion was diagnosed if the ratio of the tumour sample was 50% of the one found in HepG2. If the ratio was less than 40%, the tumour sample was considered to harbour a homozygous deletion (fig 2). To control the data for gene dosage analysis, microsatellite analysis using nine microsatellites of chromosome 9p21 was performed, as described previously. The markers used were D9S161, D9S126, D9S171, D9S172, D9S174, D9S1747, D9S1749, D9S1751, and IFNA, and were obtained from Research Genetics (Huntsville Alabama, USA) (fig 3).
Figure 1  Analysis of p14ARF and p16INK4a in three liver cell adenomas (case Nos 1, 10, and 11; same patients as in table 1). (A) p14ARF 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 p14ARF gene is methylated in case No 11 and unmethylated in case Nos 1 and 10. (B) p16INK4a 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 p16INK4a is detected in case No 1, but not in case Nos 10 and 11. (C) p16INK4a 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 p16INK4a 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 p16INK4A protein in liver cell adenoma (LCA). Case No 1 shows methylated p16INK4a and complete loss of p16INK4a (LCA cells negative for p16 protein) (original magnification×10). p16INK4a is detectable in case Nos 10 and 11 (dark reaction product within the cell nuclei) (original magnification×20 and ×40). (F) Immunostaining of p14ARF protein in LCA. Case No 1 shows unmethylated p14ARF 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 p14ARF 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.\textsuperscript{15} Exon 1β was analysed through two overlapping PCR products generated with the primer pairs P14F1 (5' TACGGGAAAGGGGCCTGGCG 3') and P14R1 (5' GCCGCGGAGTGTAACCA 3'), which generated a 245 bp product, and the primer pair P14F2 (5' GCCGCCGAGTGAGAGCT 3') and P14R2 (5' CACCGCGGTTATCTCCTC 3'), which generated a 257 bp product. The primers were labelled with \textsuperscript{32}P-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 3μl 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).

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).

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\textsuperscript{ARF} and p16\textsuperscript{INK4A}, were expressed in all cases examined; deletions were not observed. No exclusive loss of either p\textsuperscript{INK4A} or p14\textsuperscript{ARF} 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 p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} genes**

Promoter methylation of p14\textsuperscript{ARF} was present in 3/25 cases (12%). In all patients, corresponding non-neoplastic liver tissue was also analysed; no p14\textsuperscript{ARF} promoter methylation was observed in any case. Analysis of the methylation status of the adjacent p16\textsuperscript{INK4A} 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 intratumourous tissue (fibroblasts, endothelial cells, inflammatory cells). In normal LCA surrounding liver tissue, methylation of p14\textsuperscript{ARF} or p16\textsuperscript{INK4A} was not observed.

All six LCA with methylated p16\textsuperscript{INK4A} exhibited an unmethylated p14\textsuperscript{ARF} promoter. A coincidence of both p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} methylation was not found. Thus the methylation status of p14\textsuperscript{ARF} and p16\textsuperscript{INK4A} promoters does not seem to be directly related.

Real time PCR of those samples with a methylated p16\textsuperscript{INK4A} gene showed a level of methylation of approximately 75%.

All six cases with aberrant methylation of the p16\textsuperscript{INK4A} or p14\textsuperscript{ARF} gene showed complete loss of immunoreactivity (fig 1E, F) within the tumour tissue. In the 19 cases shown to lack p16\textsuperscript{INK4A} promoter methylation, nuclear staining of p16\textsuperscript{INK4A} protein was observed in nearly all LCA cells with a moderate to

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**Immunohistochemical analysis and assessment**

Immunohistochemical analysis was performed as described previously.\textsuperscript{15} In all cases tumour and non-neoplastic liver tissue was examined.
downregulated p16 detected in 22/25 tumours (fig 1D). Among the tumours with agus, and pancreas.

In early preneoplastic lesions in the lung, stomach, oesophagus, and pancreas.

Recently, aberrant methylation of the p16
\(^{INK4a}\) and p14
\(^{ARF}\) mRNA

Using specific sense primers for exon 1α and exon 1β, and a common reverse primer for exon 2, both transcripts were simultaneously amplified in a single reaction. p16
\(^{INK4A}\) mRNA was amplified in 19/25 cases and p14
\(^{ARF}\) mRNA, methylation of the corresponding promoters was observed in six and three cases, respectively.

DISCUSSION

Recently, aberrant methylation of the p16
\(^{INK4A}\) promoter has been reported not only in various types of carcinomas but also in early preneoplastic lesions in the lung, stomach, oesophagus, and pancreas.\(^{17-21}\) 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 precursor of tubulovillous colon adenoma,\(^{25,26}\) and p16
\(^{INK4A}\) in approximately 73% of tumours.\(^{24}\) In concordance with data reported for cell lines, we failed to detect specific mutations of the p14
\(^{ARF}\) or p16
\(^{INK4A}\) gene.\(^{19}\) p14
\(^{ARF}\) can also be lost by (homozygous) deletion but this loss also targets p16
\(^{INK4A}\) in the vast majority of cases.\(^{23,24}\) Only a few examples currently exist of specific p14
\(^{ARF}\) deletions that spare the remainder p16
\(^{INK4A}\) 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.\(^^{12,13,16}\) 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.\(^^{25,26}\) 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.\(^{21}\) Therefore, we hypothesise that methylation and consecutive silencing of the p16
\(^{INK4A}\) and p14
\(^{ARF}\) 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\(^{14}\) who found aberrant methylation of p16
\(^{INK4A}\) in approximately 73% of tubulovillous colon adenoma,\(^{26}\) 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,\(^{26}\) 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.

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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.
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References