Genomic homogeneity in fibrolamellar carcinomas

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Abstract

Background—Fibrolamellar carcinoma (FLC) is a variant of hepatocellular carcinoma (HCC) with distinctive clinical and histological features. To date there have been few studies on the genotypic aspects of FLC and no previous attempts have been made to use the arbitrarily primed-polymerase chain reaction (AP-PCR) technique to detect genetic alterations in this disease.

Aim—The aim of this study was to assess the degree of genomic heterogeneity of FLC using the AP-PCR technique.

Methods—A total of 50 tissue samples of primary and metastatic FLCs from seven patients were microdissected. AP-PCR amplification of each genomic DNA sample was carried out using two arbitrary primers.

Results—DNA fingerprints of the primary FLCs and all their metastatic lesions (both synchronous and metachronous disease) were identical in an individual patient. The fingerprints were different between tumours of different patients. No evidence of intratumour heterogeneity was observed.

Conclusions—Such genomic homogeneity in FLCs may explain their indolent growth. The absence of clonal evolution, which is present in other tumours (particularly HCCs), may explain the distinct behaviour in this tumour. The tumorigenic pathway and degree of somatic genomic changes in this disease may be less complex than in HCC.

Methods

Tissue samples used in this study were formalin fixed, paraffin embedded tissues of primary and metastatic FLC lesions from seven patients undergoing elective surgical resection. Details of tissue samples are summarised in table 1.

Abbreviations used in this paper: FLC, fibrolamellar carcinoma; HCC, hepatocellular carcinoma; AP-PCR, arbitrarily primed-polymerase chain reaction; LCM, laser capture microdissection.

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stained with haematoxylin and eosin for histological confirmation. The remaining 10 µm sections were stained with 0.1% toluidine blue solution for 10 minutes. Tissue samples were separately prepared by microdissection using sterile 27 gauge needles with the assistance of a dissection microscope and referenced to a section stained with haematoxylin and eosin. Deparaffinisation was achieved by incubating in xylene for 20 minutes at 55°C and then washing twice with absolute ethanol. DNA was prepared by incubating tissue in lysis buffer (10 mM Tris Cl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, proteinase K 500 µg/ml) overnight at 55°C. The lysate was subjected to three phenol/chloroform/isoamyl alcohol (25:24:1, by volume) extractions. After ethanol precipitating, washing, and drying the DNA, it was resuspended in sterile water.

LASER CAPTURE MICRODISSECTION (LCM) AND DNA PREPARATION (PATIENTS NOS 4–7)

Two 10 µm sections were serially cut from each archival block onto glass slides by microtome and stained with haematoxylin and eosin. The first section was for histological confirmation. The tissues were separately microdissected by laser capture microdissection (LCM) (Arcturus Engineering Inc., California, USA). The system employs an ethylene vinyl acetate polymer layer coated onto the underside of a rigid flat vial cap. Under the microscope, the cells of choice can be viewed through the film, and the pulsed laser beam activates a precise spot on the transfer film. At this precise location, the film immediately above the cells of interest melts and fuses with the underlying cells. The cap is lifted off the tissue and placed directly onto a 0.5 ml microfuge tube containing 50 µl of proteinase K buffer. The tube was inverted and incubated overnight at 37°C. After the incubation period, the tube was centrifuged at 13 000 rpm for five minutes and the cap was removed. Then the buffer was inactivated at 95°C for 10 minutes and the solution was ready to use as a template for PCR. Two sectors of each section were microdissected separately.

The DNA samples obtained by the microdissection techniques were qualitatively assessed; 4 µl of DNA solution were used for amplification with GAPDH primers in a total volume of 50 µl containing 50 mM KCl, 10 mM Tris Cl, pH 8.3, 1.5 mM MgCl₂, 100 pM primers, and 5 units of Taq polymerase (Bioline, London, UK). Templates were denatured for five minutes at 95°C and subjected to 35 cycles at 94°C for one minute, 55°C for one minute, and 72°C for two minutes. The PCR products were run on 1.5% agarose gel staining with ethidium bromide (data not shown). Fingerprinting of DNA samples was performed using the AP-PCR technique.

AP-PCR AMPLIFICATION

Two different arbitrary primers (AR3: 5’-GCGAATTCTAGTAGTGCAAGG-3’ and ZF3: 5’-CCCGCACGCGAGAGAAACC-3’) were used to amplify the genomic DNA of each sample. These primers have previously been shown to provide useful information on the genotypic profile of HCC. An arbitrary primer (5 µM) was labelled with γ-32P (Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase (Bioline, London, UK) at 37°C for one hour. AP-PCR amplifications were carried out using 200 ng of template DNA in a reaction mixture containing 5 µM 32P labelled arbitrary primer, 0.2 mM each dNTP (Bioline, London, UK), 10 mM Tris Cl, pH 9.2, 3.5 mM MgCl₂, 75 mM KCl, and 0.5 units of Taq DNA polymerase (Bioline, London, UK) in a final volume of 25 µl. The reactions were overlaid with mineral oil and cycled through the following temperature profile: 94°C for one minute to denature, 45°C for 30 seconds to anneal, and 72°C for 45 seconds to extend. After a final extension step of 10 minutes at 72°C, the reaction products were ethanol precipitated and resuspended in sterile water. A 5 µl aliquot of the reaction was run on a 5% polyacrylamide gel along with a 50 bp DNA ladder (Promega, Madison, WI) to assess the size of the amplified products.

Figure 1 Laser capture microdissection (LCM) of a fibrolamellar carcinoma (PLC) nodule. As no coverslip is used in LCM, the reduction in refractive index means that most light passing through the tissue is scattered which can obscure cellular detail at high magnifications. (A) A 10 µm thick paraffin embedded section of PLC tissue stained with haematoxylin and eosin. The tumour cells are separated into nests and sheets by dense lamellar bands of collagen. (B) Selected tumour cells transferred to the film of the vial caps. (C) The residual tissue section after laser capture.
five minutes for low stringency annealing of primer, and 72°C for five minutes for extension for four cycles. This temperature profile was followed by high stringency cycles: 94°C for one minute, 60°C for one minute, and 72°C for two minutes for 35 cycles, with a final extension at 72°C for 10 minutes. To ensure reproducibility, all amplifications were performed in duplicate on each sample with a Hybaid Thermoreactor (Hybaid, Middlesex, UK). The PCR product was added to 5 µl of dye mixture and was subsequently run on 8% polyacrylamide gel electrophoresis in a Model S2 sequencing gel electrophoresis apparatus (Life Technologies, Paisley, UK). After electrophoresis, the gel was transferred to a piece of Whatman 3MM paper, dried under vacuum, and exposed to x-ray film (BioMax MR, Kodak, USA) for 24–48 hours at room temperature.

Results
Comparison between the needle based and LCM techniques have been previously described. Although manual microdissection (patients Nos 1–3) was time consuming, labour intensive, and required a high degree of manual skill, it was successfully used to isolate tumour cells from sheets of collagen. The LCM technique (patients Nos 4–7) was simple, fast, required no moving parts, involved no manual microdissection or manipulations, and enabled one step transfers. Moreover, the manually microdissected cells can detach from the tip of the needle during transfer whereas cells obtained by LCM adhere to the film where they retain their morphological features and the operator can verify that the correct cells have been procured (fig 1). Tumour cells are large and polygonal. Islands of tumour are separated by dense collagenous fibrous tissue.

A total of 50 samples of primary and metastatic FLCs from seven patients were analysed (table 1). Each tumour was microdissected and tissue was taken from two sectors of each tumour (for assessment of intratumoral...
Genomic homogeneity in fibrolamellar carcinomas were the same when compared between different patients. The fingerprints of these paired reactions were identical in the same patient (figs 2, 3). The synchronous and metachronous diseases were FLCs and all their metastatic lesions (both liver, focal nodular hyperplasia, or in HCC.25 Neurotensin is expressed in fetal human liver and in FLC, but not in the adult liver, focal nodular hyperplasia, or in HCC.25 The absence of neurotensin receptor expression emphasizes the fact that neurotensin probably does not play a primary role in liver growth.

This is the first report of genomic homogeneity in FLC and metastatic lesions. Absence of the clonal evolution that is found in other tumours, particularly HCC,9 may explain the distinct behaviour of FLC. Patients with this tumour differ from those with HCC in terms of age at presentation, absence of underlying liver disease, and tumour markers. These characteristics may reflect different aetiological factors because there is no recognised association of FLC with viral hepatitis, alcohol, cirrhosis, oral contraceptives, or genetic disorders such as Wilson's disease. Tumorigenesis and degree of somatic genomic changes (rearrangements, losses, or gains) in this disease may be less complex than in HCC. None of nine patients

Figure 3 Arbitrarily primed-polymerase chain reaction (AP-PCR) analysis of primary fibrolamellar carcinomas (FLC) and their metastatic lesions from patient No 6. Autoradiograms of polyacrylamide gel electrophoresis of 32P labelled DNA fragments amplified by AP-PCR. Genomic DNA from each sample was amplified with the AR3 (A) and ZF3 (B) primers. P, primary FLC; LN, lymph nodes metastases; (number of sectors is represented at the top).

Discussion Laser assisted microdissection assists in the molecular study of FLCs because these cancers contain dense fibrous stromal components which may complicate analysis of a particular tumour cell population. The identity of the transferred cells attached to the film can be viewed and recorded by image capture. It is obviously an advantage to use microdissected cell samples in molecular analysis because the confounding effect of contaminating cells is eliminated. An increase in sensitivity of more than 50% in allelic imbalance analysis has been reported using microdissected cell populations compared with crushed tumour samples.16 Precise microdissection of phenotypically similar prostatic cancer tissue samples and analysis of the DNA for loss of heterozygosity revealed intratumoral genetic heterogeneity and provided evidence for the multifocality of tumour development in the prostate.17 AP-PCR is a highly specific technique that can generate species specific and individual diagnostic DNA fingerprints.18 This method has been applied to detect and isolate novel DNA fragments associated with somatic genomic changes in colorectal cancer, lung cancer, and astrocytoma.11-20 Moreover, it has been shown to be an uncomplicated and effective approach for scanning the genomes of tumour samples to show the evolution of differences.3

FLC has distinctive histological and clinical features. It occurs predominantly in children and young adults without a predilection for either sex, and it carries a better prognosis than the more common HCC. The indolent growth of FLC is especially obvious when recurrences develop. Even after the diagnosis of tumour recurrence, survival at one, three, and five years has been reported to be 75%, 48%, and 28% respectively.7 Several studies have attempted to associate the differences in natural history of this cancer from those of HCC. Flow cytometric analysis suggested that the DNA content in FLC is not directly related to clinical behaviour.21-22 In contrast, the overall survival rate of HCC patients with DNA aneuploid tumours was significantly poorer than that of patients with diploid tumours.23 Positive staining for neurone specific enolase, the finding of neurosecretory type granules at the ultrastructural level, and raised serum levels of neurotensin probably does not play a primary role in tumour di-

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with FLCs had p53 mutations (exons 5–8) analysed by denaturing gradient gel electrophoresis, single strand conformation polymorphism, and gene sequencing.** Moreover, in a loss of heterozygosity study using restriction fragment length polymorphism probes for various chromosomes, the rate of allelic loss in FLC was infrequent (3.6%) compared with that in HCC (16.1%).** The cell of origin of FLC remains uncertain. Whether FLC and HCC represent extremes of the spectrum of one disease entity remains uncertain. Interest-

ingly, there are few reports of the synchronous development of FLC and ordinary HCC.**

There is no defined at risk group to screen other than the young, and thus there is little prospect of improving the prognosis in this way.

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