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# DNA flow cytometry in patients undergoing liver transplantation for hepatocellular carcinoma

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**Abstract** The purpose of the study was to analyse patterns of DNA content in hepatocellular carcinomas (HCC) submitted to orthotopic liver transplantation (OLT). Paraffin-embedded archival material from 15 patients (ten men, five women, mean age 51 ± 1.78 years) transplanted in St-Roch Hospital between 1988 and 1991 was available for laboratory evaluation by flow cytometry. Five out of 15 were incidental HCC. The analysis was performed by a FACSscan flow cytometer coupled to a Hewlett-Packard computer. The cellular DNA content was defined as diploid or aneuploid in the presence of a single (DNA index of 1) or two distinct (DNA index different from 1) $G_0/G_1$ peaks, respectively. All incidental HCC (five patients) were diploid, the tumour size was 1.2 ± 0.2 cm, the number of nodules was 1.4 ± 0.24 and the mortality rate was 40 %. No death in the incidental HCC group was related to neoplastic recurrence. In the remaining ten patients transplanted for HCC, we observed 50 % diploid tumours, the tumour size was 5.2 ± 1.55 cm and the number of nodules was 2.7 ± 0.56. In this group six patients died of neoplastic recurrence (two were diploid and four aneuploid). The diameter of the neoplasm in diploid patients who died of neoplastic recurrence was over 5 cm and the number of nodules was over three. Moreover, in aneuploid patients who died of neoplastic recurrence, the diameter of the neoplasm was less than 5 cm in three cases and the number of nodules was less than three in two patients. This study indicates that incidental HCC may be a less aggressive malignancy and may have a better prognosis. In this group, no patient recurred after OLT and all tumours were diploid. Aneuploidy, tumour size (> 5 cm) and number of lesions (> 3) are prognostic indicators for neoplastic recurrence in patients transplanted for hepatocellular carcinoma.

**Key words** Hepatocellular carcinoma · Incidental hepatocarcinoma · Orthotopic liver transplantation · Nuclear DNA content (ploidy)

## Introduction

In order to design an effective therapy against a malignant tumour it is important to understand its biological behaviour. DNA flow cytometry (DNA-FCM) is an objective, quantitative technique which can measure both the degree of quantitative abnormalities of DNA content (ploidy) and the tumour proliferation rate, defined as the percentage of cells in S-phase [1]. Although many studies have investigated the relationship between DNA-FCM and the outcome of resected hepatocellular carcinoma (HCC) [2, 3], no report has been

published on transplanted HCC. The purpose of this study was to analyse patterns of DNA content in HCC submitted to orthotopic liver transplantation (OLT) and to correlate ploidy status with patients' survival.

## Patients and methods

### Patients

Retrospective DNA-FCM was performed on paraffin-embedded HCC specimens from 15 patients transplanted in St-Roch Hospital between 1988 and 1991. Ten out of 15 patients were men and the mean age was 51.33 ± 1.78 years. Five out of 15 were incidental HCC (three men, two women, mean age 50 ± 4.76 years). According to the Child-Pugh classification, 60 % of these patients had a grade C disease and 40 % had a grade B involvement. We have described in detail our technique of OLT in hepatic malignancy [4]. All patients received triple therapy immunosuppression with azathioprine, cyclosporin A and methylprednisolone [5]. Long-term passive immunoprophylaxis was realised in HbsAg-positive patients.

### Flow cytometry

Flow cytometry was performed on nuclear suspensions prepared from 50-μm sections of formalin-fixed, paraffin-embedded tissue of HCC. To determine the percentage of tumour cells in the tissue analysed, adjacent 4-μm-thick histological sections were removed before and after the 50-μm sections used for flow cytometry analysis. Briefly, the sections were dewaxed with xylene, rehydrated through 90, 80, 70 and 50 % ethanol, washed twice in deionised $H_2O$ and minced in 2 ml of 0.5 % pepsin (Sigma Chemicals, St. Louis, Mo., USA) in 0.9 % NaCl (pH 1.5) at 37 °C for 30 min. The samples were filtered through a 30-mm pore-size polyester filter and stained in propidium iodine solution [1] for 30 min at room temperature in the dark (all chemicals were from Sigma Chemicals). Before the analysis, the nuclear suspension were syringed 2–3 times through a 25 G needle to prevent nuclear clumps. A total of 40000 events was analysed on a FACSscan flow cytometer (Becton-Dickinson, San José, Calif., USA) and data on DNA content were collected and stored as list mode files using the CELLFIT software (Becton-Dickinson), without background substraction. Before each DNA-FCM procedure, the FL2 channel was calibrated with normal paraffin-embedded liver tissue. The fluorescence intensity of the $G_0/G_1$ peak of the normal cell population was set on channel 200. Given the lack of a standard procedure to calculate the DNA index (IDNA) in DNA-FCM from paraffin-embedded tissue, the $G_0/G_1$ peak with the smallest DNA content was equated with the normal diploid cells in the samples. IDNA was calculated dividing the mode channel value of the aneuploid $G_0/G_1$ peak with the mode channel value of the euploid $G_0/G_1$ peak. DNA aneuploidy was documented only if there was clear evidence of a second > $G_0/G_1$ peak. Histograms were taken into consideration only if the median CV of the diploid $G_0/G_1$ peak was equal to or less than 7 %.

### Statistical analysis

Results were expressed as means ± SEM.

## Results

All incidental HCC (five patients) were diploid, the tumour size was 1.2 ± 0.2 cm and the number of nodules was 1.4 ± 0.24. In this group we observed two deaths related to septic complication (1 and 4 months after OLT), the remaining patients are alive and free of neoplastic disease (follow-up 73, 66, 65 months from OLT). In the remaining ten patients transplanted for HCC, we observed 50 % diploid tumours, the tumour size was 5.2 ± 1.55 cm and the number of nodules was 2.7 ± 0.56. In this group the mortality rate was 90 %. Six patients died of neoplastic recurrence at 6, 12, 12, 15, 22 and 42 months after OLT (two were diploid and four aneuploid). The diameter of the neoplasm in diploid patients who died from neoplastic recurrence was over 5 cm and the number of nodules was over three. In aneuploid patients who died from neoplastic recurrence, the diameter of the neoplasm was less than 5 cm in three cases and the number of nodules was less than three in two patients. The remaining deaths, in patients transplanted for HCC, were related to septic complications (two patients) and PNF (one patient). One patient (diploid) in the HCC group is still alive and free from neoplastic disease at the time of this report (follow-up 69 months)

## Discussion

Although having a wide coefficient of variation and more cell debris, DNA-FCM using paraffin blocks has good correlation between DNA indices with flow cytometry using fresh tissue [6]. It has been reported that ploidy correlates significantly with survival rates in surgically resected HCC [2, 7, 8]. Moreover it has been shown that the DNA pattern also correlates with tumour size [7–9] and the pTNM index [8]. Nevertheless, no study has been published on DNA analysis in patients transplanted for HCC. OLT seems to be a logical treatment for HCC unaccompanied by extrahepatic disease and a growing body of evidence suggest that OLT could be the best treatment for small HCC in cirrhotic livers. Small incidental tumours found in the liver resected at OLT do not seem to recur after transplantation [10]. A recent study also suggests that patients with resectable tumours (< 3 cm) were the best candidates for OLT [11]. In our study, all incidental HCC were diploid and no recurrence of neoplastic disease was observed. Heterogeneity of the ploidy seems to reflect the biological behaviour of the tumour, usually characterised either by the existence of clones with different malignant potential or by the rapid emergence of drug resistance in the individual tumour [3]. We can speculate that the observed homogeneity of the ploidy status in association with the small size of the tumour

in incidental HCC may be responsible for the absence of neoplastic recurrence. It has been reported that tumour size, portal venous invasion, intrahepatic metastasis, atypia of tumour cells, serum αFP levels, microscopic invasion into the tumour capsule and vascular invasion, clinical stage (pTNM) are related to recurrence [12–14]. In our study we observed six neoplastic recurrences in the HCC group and two of these occurred in diploid patients. Several possible explanations can be considered for recurrence in large diploid HCC. Ploidy status estimates the quantitative changes in chromosomes by measuring the DNA content. It is difficult to identify small genetic defects in the chromosomes or the presence of small chromosomes. Likewise, balanced translocation, chromosomal rearrangement without change in chromosomal value, point mutations or deletions are difficult to detect [3]. In conclusion, the prognosis of patients seemed to be influenced by ploidy status and also by the number of tumours and tumour size. The nuclear DNA content can be precisely and rapidly evaluated by flow cytometry, from preoperative specimens [2] or from surgically resected tissues. It is expected that aggressive adjuvant therapy [13] in cases with an aneuploid DNA pattern may improve the results of the current treatment of HCC.

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