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O^6-Alkylguanine-DNA Alkyltransferase Activity in Schistosomiasis-associated Human Bladder Cancer

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O^6-Alkylguanine-DNA-alkyltransferase (ATase) activity was measured in extracts of 55 bladder tissue samples (46 tumour and nine uninvolved mucosal tissue) from Egyptian patients with schistosome-associated bladder carcinoma. Activity varied from 2.0 to 16.2 fmole ATase/μg DNA (mean ± S.D.; 5.6 ± 4.0) or from 28 to 351 fmole ATase/mg protein (117 ± 71). ATase levels in schistosome-associated bladder cancer tissues (5.6 ± 4.0 fmole ATase/μg DNA) tended to be lower than those observed in normal human bladder mucosal tissue (8.5 ± 4.4 fmole ATase/μg DNA). In a previous study (Badawi *et al.*, *Carcinogenesis*, 1992, 13, 877–881) DNA-alkylation damage (O^6-methyldeoxyguanosine) was found in 44/46 of these schistomosome-associated bladder cancer samples at levels ranging from 0.012 to 0.485 μmole O^6-MedG/mole deoxyguanosine. We now report an inverse correlation between the levels of methylation damage and ATase activity ($r = -0.67$; $P < 0.001$). These observations encourage further investigations of the possible role of environmental alkylating agents in the aetiology of early bladder cancer associated with schistosomiasis.
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INTRODUCTION

CARCINOMA OF the urinary bladder is the most abundant neoplasm in Egypt [1]. It also occurs with high frequency in different parts of Africa and in the Middle East [2]. The induction of bladder cancer has long been speculated to be in a causal association with urinary schistosomiasis caused by *Schistosoma haematobium*, which is endemic in these regions. The relationship between the two conditions has been extensively investigated, and the weight of evidence associating *S. haematobium* infection with bladder cancer is now more than sufficient to assume its validity. [1–3].

Various hypotheses have been proposed to explain the carcinogenic process induced by schistosomiasis in the bladder [1]. However, most concern has been directed towards the possible role of the *N*-nitroso compounds [1, 4], an important class of environmental carcinogens, in this process. Their presence has been demonstrated in the urine of schistosomal-infected subjects, including those with bladder cancer, at levels significantly higher than in normal individuals [4–6], and their putative roles as causative agents in the pathogenesis of some human cancer has been reviewed [7].

N-Nitroso compounds or their breakdown products can react with cellular DNA to form a complex spectrum of adducts, of which O^6-alkylguanine (O^6-RG) is thought to be the most significant as this is formed in larger amounts than the other promutagenic base O^4-alkylthymine, particularly in the case of methylating agents [8]. The persistence of O^6-RG in different tissues and cells depends strongly on the capacity of the cellular DNA repair system, and has been correlated with the cytotoxic, mutagenic, carcinogenic and other biological effects of this

group of chemical carcinogens [9]. The repair of O^6-RG, particularly O^6-methyldeoxyguanosine (O^6-MedG), is mediated by O^6-alkylguanine-DNA-alkyltransferase (ATase) in a stoichiometric autoinactivating reaction [8, 9], in which each molecule of the protein transfers one alkyl group to a cysteine residue within the molecule itself to restore the integrity of the guanine in DNA.

The constitutive level of ATase activity varies considerably, both among cells and tissues taken from different mammalian species [10–13]. In humans, comparative studies have shown that, amongst the organs studied, liver contains the highest levels of ATase activity [12, 13], while bone marrow myeloid precursors contain the lowest [13]. Although some human cells were found to be deficient in repair activity [11 ,14], various experiments have indicated that they generally have a high capacity to repair O^6-RG, and such high degrees of variation in ATase activity are to be expected in a genetically diverse population [15].

The discovery of O^6-MedG in DNA obtained from individuals suspected to be exposed to either environmentally derived [16–19] or endogenously formed alkylating agents, including those with schistosome-infected bladders from the Nile Delta region of Egypt [20], underlines the importance of understanding the DNA repair mechanism(s) which may modulate the accumulation of this promutagenic lesion in human DNA. In the present study, we have measured the levels of ATase activity in the bladder tissues of those patients with schistosomiasis, and compared them with the O^6-MedG levels previously determined [20]. This has revealed a relationship between these two parameters which contributes to the understanding of the role played by *N*-nitroso compounds in the high frequency of bladder cancer associated with schistosomiasis.

MATERIALS AND METHODS

Tissue specimens

A total of 68 human urinary bladder tissue specimens were collected. Of these, 46 samples of bladder tumour and 9 samples of bladder mucosa from adjacent regions of uninvolved tissue (i.e. with no sign of tumour invasion) were obtained during treatment by radical cystectomy of cancer patients with a history of schistosomal infection, determined at presentation for surgery. No smoking histories were available. All these tumours were malignant and were found to have originated in sites other than the trigon. These samples and a further 5 mucosal samples, obtained from patients with bladder cancer associated with factors other than schistosomiasis, were from patients living in the Nile Delta region and were collected in the Department of Urology, Faculty of Medicine, University of Alexandria. Surgical samples (8) were taken from essentially normal individuals to serve as controls and were collected at Withington Hospital, Manchester, U.K. All samples were taken from the urinary face of the bladder mucosa and were immediately frozen in dry ice. They were stored at −70°C and, in the case of the Egyptian samples, transported to Manchester in dry ice for processing.

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O^6-Alkylguanine-DNA-alkyltransferase (ATase) assay

Extracts were made by sonication of tissue (200–300 mg) in buffer I (50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, pH 8.3) for 10 s at 12 μm and 10 s at 18 μm peak-to-peak distance using a MSE sonicator. Phenylmethylsulphonylfluoride (Sigma Co., U.K.; 8.7 mg/ml ethanol) was added immediately after sonication (10 μl/ml) sonicate. Following centrifugation at 16 000 r.p.m. for 10 min at 4°C, increasing volumes of the supernatants were assayed for ATase activity in a total volume of 1.1 ml of buffer I, containing 10 μg of substrate DNA essentially as described previously [21]. The substrate was prepared by reacting calf thymus DNA with *N*-nitroso-[^{3}H]methylurea (Amersham International, 23 Ci/mmol) for 3 h at 37°C in 33 mM Tris-HCl, pH 8; 10 μg of this DNA contained 1400 cpm in 0.1 pmole O^6-MedG.

DNA and protein assay

The DNA content of each tissue extract was determined as described by Labarca and Paigen [22]. Duplicate aliquots (30 μl) of tissue extract were mixed with 2.0 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 1 ml of 1.5 μM Hoescht dye 33258 (Calbiochem-Behring, La Jolla, California, U.S.A.) in SSC was added to each tube. A standard curve was set up in duplicate using calf thymus DNA at concentrations ranging from 0.1 to 0.5 μg DNA/ml, and treated in the same manner as the samples. The fluorescence was measured at 350 nm excitation and 463 nm emission. The protein content was assayed using Bio-Rad reagent (Bio-Rad Labs, Munich, Germany) and bovine serum albumin (Sigma Co., U.K.) as standard [21].

RESULTS

ATase activity in extracts of human bladder tissues

The frequency distribution of specimens in relation to their ATase level based on both tissue protein and DNA content is shown in Figure 1. In schistosome-associated bladder cancer samples, the activity of bladder ATase showed high variations among the individuals studied. It ranged from 2.0 to 16.2 fmole ATase/μg DNA (mean ± S.D.; 5.8 ± 3.9 fmole/μg DNA) and from 28 to 351 fmole/mg protein with an overall mean of 117 ± 71 fmole/mg protein (Table 1). In the majority of the tissue samples (43 cases, 78%) the enzyme activity lay between 2 and 8 fmole ATase/μg DNA (28 to 160 fmole/mg protein) (Figure 1). Due to significant differences in cell protein content indicated by the ratio of DNA: protein in the crude cell homogenates (Table 1), all further comparisons are made on the basis of DNA content.

In the tissue specimens collected from patients with bladder cancer associated with factors other than schistosomiasis, the overall mean of ATase activity was 7.0 ± 3.6 fmole/μg DNA, and was similar to those of the schistosome-associated bladder cancer samples (5.6 ± 4.0 fmole/μg DNA)(Figure 2). The corresponding level of activity in normal human bladder tissue of European origin was 8.5 ± 4.4 fmole ATase/μg DNA, and tended to be somewhat higher than in schistosome-associated bladder cancer (Table 1).

The relationship between ATase and accumulation of O^6-MedG

O^6-MedG was previously quantified in 46 of the present series of samples [20]. The levels varied from 0.012 to 0.485 μmole O^6-MedG/mole deoxyguanosine (dG), with an overall mean of 0.134 ± 0.10 μmole/mole dG in 44/46 of the schistosome-associated bladder cancer specimens (two samples showed unde-

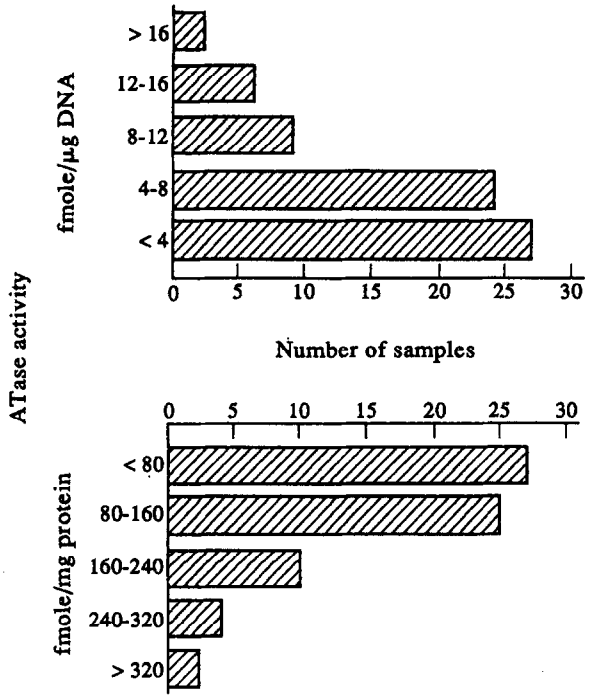

Figure 1. The frequency distribution of specimens in relation to ATase activity, expressed relative to both tissue protein and DNA content.

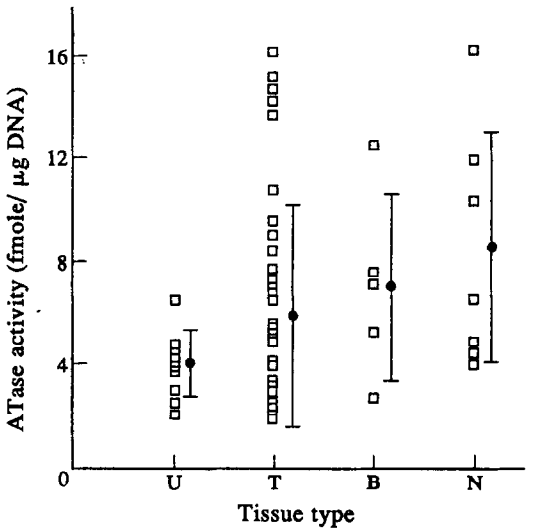

Figure 2. The profile of ATase activity in human bladder tissue samples. Data are presented individually □ for uninvolved (U) and tumour (T) tissues of schistosome-associated bladder cancer patients, the mucosa of patients with bladder cancer associated with factors other than schistosomiasis (B) and for biopsies of normal bladders (N). The overall mean values (● ± S.D.) are also shown.

Table 1. The activity of O⁶-alkylguanine-DNA alkyltransferase in human bladder tissues

Tissue origin	ATase activity (fmole per μg DNA)*	Cell homogenate (μg DNA per mg protein)
(A) Uninvolved All[a] (9)†	4.1 ± 1.3 P = 0.012; 0.024‡ (a versus i)	0.043 ± 0.018 P = 0.014; 0.042 (a versus i)
Male[b] (5)	4.1 ± 0.7	0.032 ± 0.008 P = 0.030; 0.037 (b versus c)
Female[c] (4)	4.0 ± 2.0	0.057 ± 0.017
(B) Tumour All[d] (46)	5.9 ± 4.3	0.054 ± 0.016
Male[e] (37)	6.1 ± 4.4	0.051 ± 0.027 P = 0.038; 0.048 (e versus b)
Female[f] (9)	5.0 ± 3.9	0.066 ± 0.017 P = 0.008; 0.017 (f versus e)
(A + B) All schistosome-bladder cancer[g] (55)	5.6 ± 4.0 P = 0.032; 0.044‡ (g versus i)	0.052 ± 0.025
(C) Bladder cancer (without schistosome infection)[h] (5)	7.0 ± 3.6	0.106 ± 0.023 P = 0.001; 0.001 (h versus g)
(D) European normal bladders[i] (8)	8.5 ± 4.4	0.062 ± 0.023 P = 0.017; 0.008 (i versus g)
(C + D) All non-schistosome tissues (13)	7.9 ± 4.0	0.079 ± 0.031

*Values represent mean ± S.D. †Number in parenthesis represents the number of cases. ‡P values for the logged t-test and Mann–Whitney test, respectively. [a–i], symbols used to identify groups for the purpose of comparisons of statistical significance.

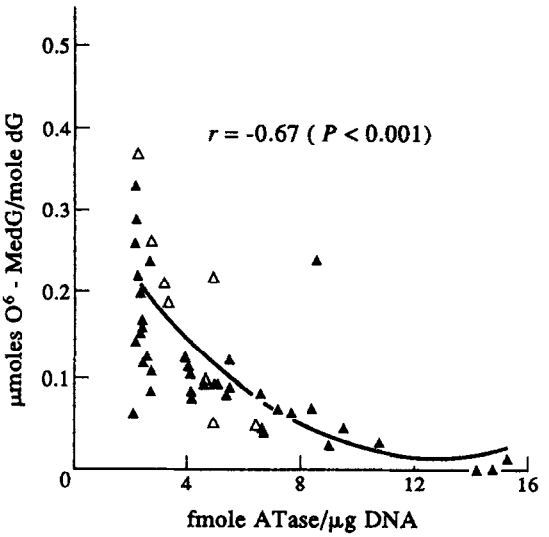

Figure 3. The relationship between ATase activity and the accumulated O^6-MedG in the studied cases. The data are expressed per μg DNA for uninvolved (△) and tumour (▲) tissue; statistical significance was calculated from the correlation coefficient of the second order regression analysis.

tectable levels of O^6-MedG). Inspection of these previous data, together with the present findings, shows a striking inverse correlation between the accumulated O^6-MedG in bladder DNA of different individuals and the corresponding ATase level. When the ATase activity, relative to accumulated O^6-MedG in the same tissue sample is compared (Figure 3), a significant negative correlation ($r = -0.67; P < .001$) is observed. A similar negative correlation ($r = -0.66; P < 0.001$) remains when activity is expressed on the basis of protein content (data not shown).

DISCUSSION

When comparing the ATase activity present in different tissues, it may be misleading to base the results on protein content which tends to be variable [14]. Therefore, more reliable estimates of the relative risk for mutagenic damage, caused by the presence of O^6-MedG, may be made relative to cellular DNA, i.e. on a per cell basis [12]. However, in order to allow comparison with earlier work (see above) both sets of data are presented.

Results obtained in the present study constitute the first report of ATase activity in human bladder tissue of individuals presenting with bladder carcinoma associated with schistosomiasis. All of the 68 tissues examined contained measurable levels of ATase activity when expressed in terms of protein or DNA. The overall mean level of ATase activity in schistosome-associated bladder cancer samples (5.6 ± 4.0 fmole ATase/μg DNA) was lower than those reported for a number of other human tissues, including liver (55.4 ± 25.8 fmole ATase/μg DNA), intestine (16.5 ± 9.1 fmole ATase/μg DNA), lung (9.1 fmole ATase/μg DNA), T-lymphocytes (7.4 ± 2.8 fmole ATase/μg DNA), colon (7.0 ± 3.9 fmole ATase/μg DNA) and brain (6.8 ± 3.5 fmole ATase/μg DNA) [12]. It was also lower than that of the normal bladder mucosa reported in the present study (8.5 ± 4.4 fmole/μg DNA; Table 1). In schistosome-associated bladder cancer samples, the average ATase level (5.6 ± 4.0 fmole ATase/μg DNA or 117 ± 73 fmole ATase/mg protein) was lower too, than those previously reported for human bladder mucosa which were 181 ± 81 and 323 ± 177 fmole ATase/mg protein from 16 samples of normal and 20 samples of neoplastic tissues, respectively [23]. There are a number of animal model carcinogenicity studies indicating that cells or tissues which express low levels of ATase activity are more susceptible to alkylating agent-induced tumours than high level expressors (see [9, 15]). It is not, therefore, unreasonable to suggest that if the ATase levels in the tumour tissues reflect those in the normal cells from which the tumour arose, the comparatively low ATase activity detected in the schistosomal-bladder tissues (see Figure 2) may possibly have made the normal cell precursors more susceptible to the carcinogenic action of agents that act by the formation of O^6-MedG in DNA [1].

Although there was a tendency for the ATase activity found in uninvolved tissues to be lower than that of the tumour tissue, this difference was non-significant (Table 1). A difference in ATase activity between the uninvolved and tumour tissue had previously been observed in a study of oesophageal tissues from Lin Xian County, China [16], in which the uninvolved tissues also had lower levels of ATase activity than the tumour tissue (190 versus 326 fmole ATase/mg protein). The lower capacity for repair in uninvolved tissues might reflect a possibly higher sensitivity of these individuals to the cancer initiating events of *N*-nitroso compounds or other environmental alkylating agents. In schistosome-infected bladder tissue, the lower ATase activity relative to other tissues [12] and to normal bladders (this study; $P \leq 0.44$) may be a consequence of a variety of causes. It could be due to the downregulation of the ATase gene by some unknown mechanism. This may be related to ethnic origin, while other possibilities include differing exposures to environmental xenobiotics and dietary factors. These questions have yet to be addressed, and the use of samples of differing origin was therefore not the ideal control. Although the European biopsy samples had a somewhat higher level of activity, they are still within the same low order of activity as the Egyptian samples. Another possible explanation for the lower ATase activity in schistosome-infected bladders is the continuous exposure to increased levels of endogenous alkylating agents in the urine and the consequent production of O^6-MedG in DNA which would then deplete ATase activity (see below).

The relatively large variation in ATase activity among the subjects studied here is not an unusual observation, since this has been demonstrated earlier in different human tissues [12, 13, 24, 25], and the seven somewhat higher values observed in the tumour series may be merely a reflection of this. However, such differences may be of great importance in the determination of individual sensitivity towards tumour initiation by alkylating agents [15]. Recent work investigating alkylating agent-induced rat stomach cancer, for example, has demonstrated that the presence of O^6-MedG in DNA, increased cell turnover and reduced ATase activity are risk factors that can act in combination and so lead to an increased frequency of tumour initiation [26]. Bladder DNA alkylation has already been demonstrated in the subjects of this study [20], and schistosomiasis is known to cause irritation and increased proliferation (i.e. promoting effects) in the bladder mucosa [1] so that ATase activity could then be a critical factor in predicting an individual's response. It should be noted that the capacity to repair O^6-MedG formed in DNA is related to both the amount of ATase present at the time of carcinogen exposure and the rate at which the ATase can be synthesised to replace that used up in the repair reaction [15]. The rate of *de novo* synthesis of ATase might differ between

individuals, either because of differences in exposure to environmental modulating factors [9–15] which might include schistosome infection (see above) or, it might be controlled primarily by genetic variation as previously suggested [24]. The identification of these factors would be of prime importance for assessment of possible risk for bladder carcinogenesis due to *N*-nitroso compounds (whether formed endogenously or ingested) during schistosomal infection.

The significant negative correlation ($P < 0.001$) observed in this study between ATase activity and the levels of O^6-MedG in bladder DNA (see results) is apparently supported by three previous reports. In oesophagus [16], tumour specimens presented higher levels of ATase activity, and these were correlated with lower average amounts of O^6-MedG than those found in normal mucosa. In addition, in human blood leucocytes taken from individuals treated with procarbazine [27] or dacarbazine [28], inverse correlations ($r = -0.76$, $P < 0.049$ and $r = -0.72$, $P = 0.008$, respectively) were observed between the rate of O^6-MedG accumulation and the pretreatment ATase activity. In these previous studies [16, 27, 28], the numbers were limited and precluded any definitive conclusion. However, taken together with the present results, they suggest that individuals may be at greater risk of accumulating O^6-MedG in their DNA as a result of constitutively low ATase levels, and this may be exacerbated by O^6-MedG arising from environmental exposures. Low residual levels of O^6-MedG in DNA may not necessarily result from the expression of high levels of ATase. They could arise from a lower capacity for the metabolism of environmental alkylating agents or possibly, from the presence of ATase deficient cells among a population of ATase proficient cells. Also, certain regions of DNA, e.g. nuclear matrix DNA, may be repaired of O^6-MedG much less efficiently than the bulk genomic DNA [29]. It is also possible that, in some individuals, environmentally induced O^6-MedG may occur in sufficient quantities to substantially deplete these relatively low constitutive levels of ATase activity. The extent to which our data represents a steady state between ATase production or the extensive and transient depletion of ATase due to 'pulse(s)' of DNA alkylation has yet to be established.

For the urinary bladder, a substantial amount of evidence now exists which supports the association of exposure to *N*-nitroso compounds and the development of carcinoma in schistosome-infected patients [1]. Significant amounts of *N*-nitroso compounds and their precursors (see [1]) are found in the Egyptian environment, and increased levels of endogenous formation of this group of chemical carcinogens occur in the urine of schistosomal infected subjects [4–6]. The bladder mucosa has the capacity to metabolise these agents [1], and alterations in the activities of the hepatic-carcinogen metabolising systems following the schistosomal infection have been noted [1]. In mice infected with *Schistosome mansoni* [30], O^6-MedG has been detected at levels similar to those found in the infected bladders of patients with schistosomiasis [20]. The high frequency of detection of O^6-MedG observed in the present study in comparison with normal bladder and other tissues suggest that there would be a higher than normal sensitivity to bladder carcinogenesis. Finally, the continuous friction of the calcified eggs against the hyperplastic urothelium, which invariably accompanies urinary schistosomiasis, along with the preceeding factors might be expected to combine to create an ideal environment for the mucosa to develop precancerous lesions (see [1]); all of these factors could contribute to the increased incidence of bladder cancer associated with schistosomiasis.

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Treatment of Unresectable Hepatocellular Carcinoma With a Combination of Human Recombinant α-2b Interferon and Doxorubicin: Results of a Pilot Study

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Based on the *in vitro* and *in vivo* potentiation of the cytotoxic activity of chemotherapeutic agents by the interferons, a pilot study combining human recombinant α-2b interferon (IFN) and doxorubicin was conducted for the treatment of unresectable, histologically proven hepatocellular carcinoma. Between March 1988 and May 1990, 21 patients (median age: 60 years, range: 29–76) entered the study. The dose of doxorubicin was fixed at 35 mg/m², every 3 weeks. The dose of α-2b IFN was 6 million U/m² per day, 5 days a week. 3 patients (14%) obtained a partial response lasting 11, 16 and 30 months, and 1 had a stable disease during 8 months. The other 17 patients died within a median survival time of 4 months. All patients experienced flu-like symptoms. 7 patients experienced WHO grade III-IV haematological toxicity. We conclude that the association of α-2b IFN and doxorubicin is feasible, with respect to the use of doxorubicin at an inferior dose level than the same agent used without IFN. The response rate is comparable to that observed with doxorubicin used alone. Further phase I studies and randomised trials are required to confirm the role of this regimen in the treatment of unresectable hepatocellular carcinoma.

Key words: hepatocellular carcinoma, doxorubicin, interferon
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INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) generally has a very poor prognosis because it is usually present at an advanced stage [1]. Surgical resection offers the best hope of cure [1]. Even when the tumour is resectable, an operative morbidity rate between 11% and 25% is reported [1–4] and then, the mean 5-year survival rate after complete resection is about 25%. Only a few patients have, in fact, resectable tumours because, in most cases, tumours involve the two hepatic lobes. Some patients have initially metastatic diseases. Moreover, most patients have liver