Xenografts of Human Hepatocellular Carcinoma: A Useful Model for Testing Drugs
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Abstract

Purpose: Our aims were to establish and characterize primary human hepatocellular carcinoma xenografts. They were used to screen new drugs and improve our current treatment regimens used in hepatocellular carcinoma.

Experimental Design: Primary hepatocellular carcinomas were used to create the xenografts. Western blotting was used to determine the changes in proteins in these xenografts before and after therapies. Apoptotic and cell proliferation were analyzed by immunohistochemistry.

Results: Seven lines of xenografts were established from primary human hepatocellular carcinomas. Lines 4-1318, 2-1318, 2006, and 26-1004 grew rapidly in severe combined immunodeficient (SCID) mice and doubled its volume every 48 to 72 hours. Series 5-1318 (5-1318, 30-1004, and 29-1104) grew relatively slowly in SCID mice and required 6 to 10 days to double its tumor volume. Western blot analysis revealed that the growth rate of these xenografts was associated with abnormal expression of proteins associated with the cell cycle, signaling pathways, and tumor suppressor genes. Although hepatocellular carcinoma xenografts expressed the receptors for androgens, estrogens, and progesterone, their growth rate was not affected by either castration or sex steroid hormone supplementation. Cisplatin, oxaliplatin, vitamin D analogue EB1089, and Iressa had no effects on the growth rate in SCID mice. Although 5-fluorouracil exerted mild growth inhibition of these xenografts, i.p. delivery of 2-chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU) or doxorubicin resulted in a significant growth inhibition. Doxorubicin-induced growth suppression was associated with elevation of p53 and p21\(^{Cip1/Waf1}\). In addition to up-regulation of p53 and p21\(^{Cip1/Waf1}\), SarCNU also increased the levels of phosphorylated cdc-2 at Tyr\(^{15}\).

Conclusion: Hepatocellular carcinoma xenografts are powerful tools for screening drugs and SarCNU may be useful in the treatment of this fatal disease.

Hepatocellular carcinoma comprises ~40% of all cancers in Southeast Asia, Japan, and Africa (1). The annual incidence is ~626,000 in the year 2002 (2). The disease is associated with environmental exposure to hepatitis B and C viruses (HBV and HCV) and aflatoxin B1 (3, 4). Patients with poorly differentiated tumors or cirrhosis in their tumor sample typically exhibit poorer survival (5). Surgery is the only established potentially curative therapy for hepatocellular carcinoma. Unfortunately, 85% to 90% of hepatocellular carcinoma is inoperable.

Materials and Methods

Reagents. Antibodies against pRB, α-tubulin, cyclin A, cyclin B1, p53, cyclin D1, CDK-4, CDK-6, cdc-2, c-myc, p21\(^{Cip1/Waf1}\), p27,
androgen receptor, E2F-1, E2F-2, E2F-3, E2F-5, extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein/ERK kinase 1/2 (MEK1/2), GSK-3α/β, CDK-2, and MEK-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cleaved caspase-3, epidermal growth factor-R, ErbB-2, and ErbB-3 antibodies and phosphorylation-specific antibodies against epidermal growth factor receptor (EGFR; Tyr1045), ErbB-2 (Tyr1222), GSK-3α/β (SerSer9/9), MEK1/2 (Ser72/27/29), ERK (Thr202/Thr204), pRB (Ser807/811), and pBB (Ser603) were obtained from Cell Signalling Technology (Beverly, MA). Antibodies against estrogen receptor-a, PTEN, Ki-67, p82/130, MDM-2, and progesterone receptor were obtained from NeoMarkers (Fremont, CA). Horseradish peroxidase–conjugated secondary antibodies were supplied by Pierce (Rockford, IL). Mouse anti-human HBV and HCV antibodies were from Novocastra Laboratory, Ltd. (Newcastle upon Tyne, United Kingdom). Chemiluminescent detection system was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

17-β-Estradiol, progesterone, and dihydrotestosterone were obtained from Sigma-Aldrich (Singapore). Cisplatin, Fluorouracil Injection BP, and oxaliplatin were from Faulding Pharmaceuticals Pte, (Warwickshire, United Kingdom). Iressa was a gift from AstraZeneca Pharmaceuticals (Cheshire, United Kingdom). Doxorubicin was from Pharmacia & Upjohn S.P.A. (Rydalmere, Australia). 2-Chloroethyl-3-sarcosinamide-1-nitrosourea (SarCNU) and Seocalcitol (EB1089) were a gift from the National Cancer Institute (Bethesda, MD) and Leo Pharmaceutical (Ballerup, Denmark), respectively.

Establishment of hepatocellular carcinoma xenografts. Prior written informed consent was obtained from all patients and the study received ethics board approval at the National Cancer Centre of Singapore as well as the Singapore General Hospital. All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the NIH. They were provided with sterilized food and water ad libitum and housed in negative pressure isolators with 12 hours light/dark cycles.

Between January to September 2000 with the assistance of the Department of General Surgery, Singapore General Hospital, eight primary hepatocellular carcinomas were obtained intraoperatively during liver resection at the Singapore General Hospital. The diagnosis of hepatocellular carcinoma was confirmed by histology in all cases. Hepatocellular carcinoma xenografts were carried out with mice homozygous for the severe combined immune deficient (SCID) mutation (SCID/SCID, The Jackson Laboratory, Harbor, ME). Briefly, primary hepatocellular carcinoma tumors were placed in RPMI 1640 in an ice bath in the surgical site. Thin slice of tumor were diced into 2 to 3 mm³ pieces and washed thrice with RPMI 1640. They were minced into fine fragments that would pass through an 18-gauge needle. They were then placed in 70% ethanol, and transferred to a laminar flow hood. The tissue mixture was s.c. injected in both flanks of 8-week-old male SCID mice. For each tumor, six to eight mice were used. Growth of established tumor xenografts was monitored at least twice weekly by vernier caliper measurement of the length (a) and width (b) of tumor. Tumor volumes was calculated as \(a \times b^2 / 2\). For serial transplantation, tumor-bearing animals were anesthetized with diethylether and sacrificed by cervical dislocation. Animals were placed immediately in an ice water bath for 2 minutes. They were then dipped in and out of 10% Clorox solution for 2 minutes, washed in four changes of ice water, placed in 70% ethanol, and transferred to a laminar flow hood for dissection. Tumors were minced under sterile conditions and injected in successive SCID mice as described above.

Systemic and hormonal treatments. 2-1318 and 5-1318 lines (passages 9-10) were implanted as described above. Mice bearing hepatocellular carcinoma xenografts were treated with various drugs or hormones starting on day 4 after tumor implantation as described below. At the end of each experiment, body weight and tumor weight were recorded. Tumors were harvested, frozen in liquid nitrogen, and fixed in buffer containing 10% formalin and embedded in paraffin for histologic study. Each experiment was repeated at least twice.

We have shown that treatment of hepatoma cells with SarCNU resulted a G2-M cell cycle arrest (13). To investigate the effects of SarCNU on hepatocellular carcinoma xenografts, SarCNU was dissolved in 0.01 mol/L sodium acetate buffer (pH 4.9) at an appropriate concentration. Mice were i.p. injected daily with either 100 µL of fresh prepared vehicle \((n = 14)\) or 60 mg/kg \((n = 14)\) or 80 mg/kg \((n = 14)\) of SarCNU for 5 days as described (14). Animals were sacrificed on day 21 after SarCNU treatment.

It has been reported that ZD1839 (Iressa) inhibited growth of the implanted hepatocellular carcinoma and intrahepatic metastasis by 50% (15). To examine the antineoplastic activity of Iressa on hepatocellular carcinoma, Iressa was suspended in sterile distilled water and orally given to mice bearing hepatocellular carcinoma xenografts \((n = 7)\) daily at a dose of 200 mg/kg for 3 weeks. Control mice \((n = 7)\) received only water. Animals were sacrificed 6 hours after the last dose.

Long-term exposure to steroid hormones has been implicated in the development of hepatocellular carcinoma (reviewed in refs. 16, 17). To study the effects of steroid hormones on the growth of hepatocellular carcinoma xenografts, mice bearing hepatocellular carcinoma tumors were either sham castrated or castrated. Castrated mice were then implanted with either silastic tubes \((0.078 \text{ in.}, \text{ID}, \text{Dow Corning})\) containing 17-β estradiol \((n = 7)\) or progesterone \((n = 7)\) or dihydrotestosterone \((n = 7)\) on the back of their necks. Control mice \((n = 7)\) underwent the same surgical implantation with empty silastic tubes. The release rate of 17-β-estradiol, progesterone, and dihydrotestosterone from the implants was 2.4, 7.2, and 7.2 µg/d. Animals were sacrificed 27 days after hormonal implantation.

Because doxorubicin, cisplatin, and 5-fluorouracil (5-FU) are the most studied individual agents for inoperable hepatocellular carcinoma \((8-10, 18)\), the effects of these drugs were tested on hepatocellular carcinoma xenografts. Xenograft-bearing mice were treated with doxorubicin \((1 \text{ mg/kg;} n = 12)\), 5-FU \((25 \text{ mg/kg;} n = 12)\), oxaliplatin \((12.5 \text{ mg/kg;} n = 6)\), and cisplatin \((6 \text{ mg/kg;} n = 6)\). Doxorubicin was given by i.v. injection every 3 days for 3 weeks; 5-FU was i.p. injected on days 1, 7, and 14 for 3 weeks; oxaliplatin and cisplatin were given by i.v. injection weekly for 3 weeks. Animals were sacrificed 3, 7, and 7 days after the last injection for doxorubicin, oxaliplatin, cisplatin, and 5-FU, respectively.

Long-term administration of vitamin D analogue EB1089 caused a significant decrease in hepatocellular carcinoma incidence in C3H/Scid mice (19). To examine the antineoplastic activity of vitamin D, EB1089 was suspended in propylene glycol: 0.05 mol/L NaPO₄ \((80:20)\) at a concentration of 10 µg/mL. Mice bearing hepatocellular carcinoma xenografts (eight per group) received daily i.p. injections of either vehicle or 0.5 µg EB1089 or 1.0 µg EB1089 per kg body weight for 3 weeks as described (19). Animals were sacrificed 24 hours after the last injection.

Western blotting. For the protein expression profile, 4-1318 (passage 9), 2-1318 (passage 8), 26-1004 (passage 9), 5-1318 (passage 7), 29-1318 (passage 7), and 30-1004 (passage 8) lines were used. Total proteins \((100 \mu g)\) were analyzed by Western blotting as previously described (20). All primary antibodies were used at a final concentration of 1 µg/mL. The blots were then visualized with a chemiluminescent detection system as described by the manufacturer.

Histology and immunolocalization of HBV, HCV, Ki-67, cleaved caspase-3, phosphorylated p53 at Ser15, and p21Cip1/Waf1. Five-micrometer sections were cut, dewaxed, and then rehydrated and stained with H&E as described (20). For immunohistochemistry, sections were incubated overnight with antibodies against cleaved caspase-3, human HBV, human HCV, Ki-67, phosphorylated p53 at Ser15, and p21Cip1/Waf1 at 4°C as described (20). The slides were examined and pictures were taken using an Olympus BX60. Sections known to stain positively were incubated in each batch and negative controls were also prepared by replacing the primary antibody with preimmune sera. For Ki-67 and p21Cip1/Waf1, only nuclear immunoreactivity was considered positive. The labeling index was obtained by counting...
the number of labeled cells among at least 500 cells per region and was then expressed as percentage values.

**Statistical analysis.** For quantitative analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated, and the amount of α-tubulin was normalized. Differences in the levels of protein under study, tumor incidence, and tumor volume among groups were analyzed by ANOVA.

**Results**

The primary hepatocellular carcinoma tissues obtained by resection were finely minced, mixed with Matrigel, and injected s.c. into male SCID mice on the day of surgery. Seven of eight primary tumors could be grown as xenografts. Clinical characteristics of the primary tumors and viral infection were shown in Table 1. Lines 4-1318, 2-1318, 2006, and 26-1004 were first detected 8 to 12 weeks posttransplantation and grew sufficiently rapidly to allow a second passage and histologic documentation of hepatocellular carcinoma in the second mouse after serial transplantation. Lines 5-1318, 29-1104, and 30-1004 appeared ~22, 18, and 20 weeks after initial transplantation, respectively. They were transplanted into a second mouse 32 weeks after its first transplantation. Although six of seven hepatocellular carcinoma lines were derived from HBV-positive hepatocellular carcinoma (Table 1), none of the hepatocellular carcinoma lines retained viral replication through the passages in mice. The mice that received 2006-7 hepatocellular carcinoma were observed for 1 year after transplantation, and none of the tumors developed into serially transplantable xenografts.

The xenografts were divided into the 1318 (4-1318, 2-1318, 2006, and 26-1004) and 5-1318 (5-1318, 29-1104, and 30-1004) series based on their histology, protein profile, and growth characteristics (Figs. 1 and 2). As shown in Fig. 1A, 1318 grew rapidly and the tumor volume doubled every 2 to 3 days. 5-1318, on the other hand, grew slowly and needed ~6 to 10 days to double its volume. Autopsies of mice bearing 1318 and 5-1318 xenografts for 2 months revealed no evidence of metastases in the liver, lung, kidney, or brain. Figure 1B and C shows that the 5-1318 xenografts consisted of trabeculae with intervening acinar-like spaces and had a pseudoglandular pattern. The 1318 xenografts consisted of parallel cords of hepatocytes in a predominantly well-differentiated pattern, polyhedral cells, and large hyperchromatic nuclei, and a high rate of mitosis (Fig. 1D and E).

Because cell cycle regulators play an important role in the development and progression of hepatocellular carcinoma (reviewed in ref. 21), the levels of cyclins and CDKs in the xenografts were examined. As shown in Fig. 2A, cyclin D1, cdc-2, CDK-6, and to a lesser extent CDK-2, expression was significantly elevated in 1318 compared with 5-1318 series of xenografts (P < 0.01). The levels of cyclin A, p21CIP1/WAF1, and cyclin B1 were not different between the two series. CDK-4 appeared as a single band in 1318 but was in a doublet form in 5-1318. The levels of p27KIP1 and c-myc were significantly lower in 1318 than 5-1318 (P < 0.01; Fig. 2A).

Because phosphorylation of MEK1/2 and ERK1/2 was required for the proliferation and survival of hepatoma cells (22), we investigated whether the differences in growth rate between the 1318 and 5-1318 series of xenografts were associated with the activation of MEK-ERK pathway. Figure 2A shows that 1318 expressed higher levels of phosphorylated MEK1/2 than 5-1318 (P < 0.01). Surprisingly, phosphorylated ERK1/2 was significantly lower in 1318 than in 5-1318 (Fig. 2A; P < 0.01). The levels of phosphorylated GSK-3β (Ser21/9) were also elevated in 1318 (Fig. 2A). The levels of vitamin D receptor, insulin-like growth factor-I receptor and ErbB-3 (data not shown), EGFR, phospho-EGFR (Tyr1045), ErbB-2, and phospho-ErbB-2 (Tyr1067, Fig. 2B) were found to be similar between the 1318 and 5-1318 series.

Figure 2B shows that although MDM-2 expression was high, the levels of p53 and pRb2 p130 in 1318 were significantly lower than those seen in 5-1318 (P < 0.01). A doublt of ~105 to 120 kilodaltons was detected by pRb antibody in 1318 but only a 105 kilodalton species was detected in 5-1318. Although the total amount of pRb in 1318 series was slightly higher than that found in 5-1318, pRb in 1318 was hyperphosphorylated at Ser277 and Ser380/381 (Fig. 2B). Although PTEN, E2F-2, and E2F-3 levels were similar, E2F-1 expression was high in the 1318 series but barely detectable in 5-1318. A single band of E2F-5 was detected in 1318 but it appeared as a doublet in 5-1318 (Fig. 2B). Figure 2B also shows that both the 1318 and 5-1318 series expressed estrogen receptor-α, progesterone receptor, and androgen receptor. 5-1318 had an ~5-fold higher progesterone receptor than 1318 (P < 0.01). Because the protein profile and growth behavior were quite similar among the lines within the same series, we selected the 2-1318 and 5-1318 lines to represent the 1318 and 5-1318 series for our subsequent studies. Both of them express wild-type p53.

Because long-term exposure to steroid hormones has been implicated in the development of hepatocellular carcinoma (16, 17), the effects of castration, testosterone, progesterone, and estrogens on the growth of hepatocellular carcinoma...
xenografts were examined. Similar to EGFR inhibitor Iressa and vitamin D analogue EB1089, castration and steroid hormones had no effect on cell proliferation and apoptosis as determined by Ki-67 and cleaved caspase-3 staining (Table 2). A significant loss in body weight was noticed in the EB1089- and Iressa-treated groups compared with vehicle ($P < 0.05$).

Although oxaliplatin and cisplatin had no effects on the growth of hepatocellular carcinoma xenografts, doxorubicin, and to a lesser extent 5-FU, significantly inhibited them ($P < 0.01$). The magnitude of growth inhibition was $\sim 20\%$ and $40\%$ upon treatment with 5-FU and doxorubicin, respectively (Fig. 3B). Immunohistochemistry showed that the Ki-67 index in vehicle- and doxorubicin-treated groups was $17.4 \pm 5.6\%$ and $7.8 \pm 2.5\%$, respectively ($P < 0.01$). The cleaved caspase-3 index was $7.4 \pm 2.6\%$ and $14.8 \pm 3.5\%$, respectively ($P < 0.01$). A significant decrease in body weight was seen in the doxorubicin-treated group compared with controls (Fig. 3A; $P < 0.01$). The antineoplastic activity of doxorubicin but not 5-FU was associated with significant upregulation of p53 and p21$^{Cip1/Waf1}$ ($P < 0.01$; Fig. 3C).

Because treatment of hepatoma cells with SarCNU resulted in G2-M cell cycle arrest (13), effects of SarCNU on tumor growth of hepatocellular carcinoma xenografts and animal toxicity were investigated. For 2-1318, tumor formation at the end of the experiment was 100% (14 of 14), 85% (12 of 14), and 71% (10 of 14) in the vehicle, and 60 and 80 mg SarCNU–treated groups, respectively. For 5-1318, the tumor incidence was 100% (14 of 14), 78.5% (11 of 14), and 64% (9 of 14) in the vehicle, and 60 and 80 mg SarCNU–treated groups, respectively. Differences in tumor incidence between the control and SarCNU-treated groups were statistically significant at $P < 0.01$. Figure 4A shows that the body weight...
was significantly decreased in the 80 mg SarCNU–treated group ($P < 0.05$). Figure 4B shows that SarCNU inhibited the growth of 2-1318 xenografts in a dose-dependent manner ($P < 0.01$). A similar growth pattern was obtained when 5-1318 xenografts were treated with SarCNU (data not shown). As seen in both 2-1318 and 5-1318, the final tumor weight in the SarCNU-treated groups was significantly reduced compared with the vehicle-treated one ($P < 0.01$; Fig. 4C). The Ki-67 index in vehicle-treated, and 60 and 80 mg SarCNU–treated groups was $18.4 \pm 5.6\%$, $5.4 \pm 1.8\%$, and $1.8 \pm 0.8\%$, respectively ($P < 0.01$). The cleaved caspase-3 index in vehicle-treated, and 60 and 80 mg SarCNU–treated groups was $8.4 \pm 1.6\%$, $7.4 \pm 2.8\%$, and $8.3 \pm 1.7\%$, respectively. Both Ki-67 and cleaved caspase-3 indexes were significantly reduced by SarCNU treatment ($P < 0.01$).

Because SarCNU inhibited the growth of hepatoma cells by up-regulation of p53, p21Cip1/Waf1, and phosphorylated cdc-2 at Tyr15 (13), we investigated if similar mechanisms also operated in hepatocellular carcinoma xenografts following SarCNU treatment. Figure 5 shows that p53, phosphorylated p53 at Ser15, p21Cip1/Waf1, and phosphorylated cdc-2 at Tyr15 in the SarCNU-treated 2-1318 xenografts were significantly elevated ($P < 0.01$). Immunohistochemistry revealed that about 75% of the cells in SarCNU-treated 2-1318 tumors (Fig. 5I) were stained for phosphorylated p53 at Ser15 compared with 0.1% of cells in the vehicle-treated tumors (Fig. 5H). Similar results were obtained when 5-1318 xenografts were used (data not shown). Cells in the SarCNU-treated (Fig. 5K) but not vehicle-treated (Fig. 5J) 2-1318 xenografts exhibited nuclear staining for p21Cip1/Waf1. The results indicate that SarCNU was able to induce p53 and p21Cip1/Waf1 accumulation and the phosphorylation of p53 at Ser15 in vivo.

Discussion

This study is the first report of a series of primary hepatocellular carcinomas in which a significant proportion has grown significantly in SCID mice such as to allow serial transplantation. The xenografting of primary hepatocellular carcinoma seems to be the only currently available means that will permit the propagation of a significant proportion of these primary carcinomas. Even with the methods described in...
Table 2. Effects of cisplatin, oxaliplatin, 17-β-estradiol, progesterone, dihydrotestosterone, EB1089, and Iressa on Ki-67 index, apoptosis, and growth of hepatocellular carcinoma xenografts

<table>
<thead>
<tr>
<th>Xenografts</th>
<th>Treatments</th>
<th>No.</th>
<th>Body weight (g)</th>
<th>Tumor weight (mg)</th>
<th>Ki-67 index</th>
<th>Cleaved caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1318</td>
<td>Vehicle</td>
<td>6</td>
<td>24.35 ± 0.56</td>
<td>1434.7 ± 470</td>
<td>11.2 ± 2.6</td>
<td>6.4 ± 2.1</td>
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<td>Cisplatin</td>
<td>6</td>
<td>20.39 ± 1.8</td>
<td>1306.3 ± 290</td>
<td>10.3 ± 2.0</td>
<td>6.8 ± 3.1</td>
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<td>Oxaliplatin</td>
<td>6</td>
<td>22.16 ± 1.5</td>
<td>1299.7 ± 270</td>
<td>9.6 ± 1.8</td>
<td>7.3 ± 2.4</td>
</tr>
<tr>
<td>5-1318</td>
<td>Vehicle</td>
<td>6</td>
<td>23.61 ± 0.48</td>
<td>718.5 ± 169</td>
<td>7.1 ± 1.7</td>
<td>4.6 ± 1.7</td>
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<td>Cisplatin</td>
<td>6</td>
<td>22.18 ± 1.04</td>
<td>654.7 ± 123</td>
<td>6.8 ± 1.3</td>
<td>5.2 ± 1.4</td>
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<td></td>
<td>Oxaliplatin</td>
<td>6</td>
<td>22.50 ± 1.23</td>
<td>616.8 ± 144</td>
<td>7.2 ± 1.5</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td>2-1318</td>
<td>Vehicle</td>
<td>8</td>
<td>25.1 ± 0.87</td>
<td>1369.3 ± 289</td>
<td>12.4 ± 2.4</td>
<td>6.7 ± 1.8</td>
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<td>EB1089 (0.5 μg/kg/d)</td>
<td>8</td>
<td>18.97 ± 1.80*</td>
<td>1163.1 ± 261</td>
<td>11.0 ± 1.8</td>
<td>7.8 ± 1.9</td>
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<td>EB1089 (1 μg/kg/d)</td>
<td>8</td>
<td>17.23 ± 1.42*</td>
<td>1089.3 ± 187</td>
<td>11.2 ± 1.9</td>
<td>8.5 ± 2.4</td>
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<td>5-1318</td>
<td>Vehicle</td>
<td>8</td>
<td>23.9 ± 1.03</td>
<td>802.4 ± 180</td>
<td>8.1 ± 1.85</td>
<td>4.9 ± 1.5</td>
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<td>EB1089 (0.5 μg/kg/d)</td>
<td>8</td>
<td>18.06 ± 1.26*</td>
<td>756.2 ± 118</td>
<td>7.3 ± 1.6</td>
<td>5.1 ± 1.6</td>
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<td>EB1089 (1 μg/kg/d)</td>
<td>8</td>
<td>16.83 ± 1.21*</td>
<td>708.4 ± 121</td>
<td>8.0 ± 1.7</td>
<td>7.3 ± 2.2</td>
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<td>2-1318</td>
<td>Vehicle</td>
<td>6</td>
<td>24.24 ± 1.03</td>
<td>1674.5 ± 229</td>
<td>13.8 ± 3.1</td>
<td>8.1 ± 2.4</td>
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<td>Iressa (200 mg/kg)</td>
<td>7</td>
<td>20.87 ± 0.94*</td>
<td>1681.4 ± 252</td>
<td>12.6 ± 2.5</td>
<td>6.5 ± 1.9</td>
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<tr>
<td>5-1318</td>
<td>Vehicle</td>
<td>6</td>
<td>23.54 ± 1.12</td>
<td>763.5 ± 144</td>
<td>9.5 ± 2.6</td>
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<td>Iressa (200 mg/kg)</td>
<td>7</td>
<td>20.32 ± 0.81*</td>
<td>750.6 ± 136</td>
<td>8.4 ± 1.9</td>
<td>6.9 ± 1.8</td>
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<td>Sham</td>
<td>7</td>
<td>27.5 ± 1.35</td>
<td>1387.6 ± 216</td>
<td>14.3 ± 3.5</td>
<td>7.8 ± 1.6</td>
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<tr>
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<td>C</td>
<td>7</td>
<td>29.8 ± 0.87</td>
<td>1216.5 ± 179</td>
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<td>C + E2 (2.4 μg/d)</td>
<td>7</td>
<td>25.1 ± 108</td>
<td>1651.2 ± 212</td>
<td>15.8 ± 2.9</td>
<td>5.8 ± 2.1</td>
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<td>C + P4 (7.2 μg/d)</td>
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<td>29.8 ± 0.89</td>
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<td>13.1 ± 2.3</td>
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<td>C + DHT (7.2 μg/d)</td>
<td>7</td>
<td>30.6 ± 1.07</td>
<td>1191.8 ± 165</td>
<td>9.8 ± 1.6</td>
<td>9.3 ± 2.4</td>
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<td>5-1318</td>
<td>Sham</td>
<td>7</td>
<td>25.64 ± 0.87</td>
<td>792 ± 134</td>
<td>8.1 ± 2.4</td>
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<td>7</td>
<td>27.6 ± 0.88</td>
<td>806 ± 114</td>
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<td>874 ± 118</td>
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<td>C + P4 (7.2 μg/d)</td>
<td>7</td>
<td>26.4 ± 0.89</td>
<td>763 ± 87</td>
<td>10.3 ± 2.7</td>
<td>5.5 ± 1.8</td>
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<td></td>
<td>C + DHT (7.2 μg/d)</td>
<td>7</td>
<td>27.5 ± 1.06</td>
<td>750 ± 90</td>
<td>8.9 ± 3.4</td>
<td>6.8 ± 2.6</td>
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NOTE: 2-1318 and 5-1318 xenografts were established as described in Materials and Methods. Mice bearing tumors were treated with either vehicle or the indicated compounds as described in Materials and Methods. Differences in final body weight and final tumor weight among the treatment groups were significant at $P < 0.05$, as analyzed by ANOVA.

Abbreviations: C, castration; E2, 17-β-estradiol; P4, progesterone; DHT, dihydrotestosterone.

*P < 0.05.

detail here, we cannot be precise in predicting what proportion of hepatocellular carcinoma will be serially transplantable in SCID mice. The unique feature of these lines of hepatocellular carcinoma xenografts is that the implanted tumor tissue still retains most of its normal architecture and function and thereby facilitates tumor cells to grow. This model more accurately reflects the in vivo situation than cancer cell lines.

Although six of seven primary hepatocellular carcinoma yielding these xenografts are positive for HBV (Table 1), the viral replication is lost through the passages in mice. Although xenograft series 5-1318 grow much slowly than series 1318, there are no significant differences in tumor recurrence or patient survival in the two groups of patients yielding them. Our data suggest that their growth behavior is positively correlated with the levels of phosphorylated MEK1/2, phosphorylated GSK-3α/β, hyperphosphorylation of pRB, cdc-2, cyclin D1, CDK-2, CDK-6, MDM-2, and E2F-1, and inversely correlated with the levels of p27, p53, pRb2/p130, c-myc, and progesterone receptor. The observed slow growth of 5-1318 may be due to its high basal expression of p53, p27, hypophosphorylated pRB, and pRb2/p130. This hypothesis is supported by our recent study showing that pRb2/p130 acts as a tumor suppressor gene (23) and that phosphorylation of MEK1/2 is required for the growth and survival of hepatoma cells (22).

Overexpression of cyclin D1 and E2F-1, and inactivation of pRB by phosphorylation, may also contribute to the observed rapid growth rate of the 1318 series because hyperphosphorylation of pRB allows E2F-1 to be constitutively active. This may lead to rapid cell proliferation and enhanced hepatocellular carcinoma aggressiveness. Because cyclin D1/CDK-4 preferentially phosphorylates pRB at Ser780 (24) and Ser792 (25, 26), the hyperphosphorylation of pRB seen in 1318 could be due to the activation of cyclin D1/CDK-4 and/or cyclin D1/CDK-6 complexes. In the present study, we observed that 1318 overexpress cyclin D1 and grow faster than 5-1318, which has low levels of cyclin D1. This observation is consistent with previous studies demonstrating that cyclin D1 expression is sufficient to promote cell cycle progression (27), to initiate hepatocellular carcinogenesis (28), and to be associated with aggressive forms of hepatocellular carcinoma (21, 29–31). The molecular mechanisms responsible for cyclin D1 overexpression in the 1318 series are not known. It is possible that activated MEK1/2 is


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responsible for the elevation in cyclin D1 in 1318 as previously described (32).

In this study, high levels of phosphorylated MEK1/2 were observed in fast-growing xenografts, suggesting that MEK activation may be linked with the malignant progression of liver cancer. This hypothesis is supported by our recent study showing that phosphorylation of MEK1/2 is also detected in a high proportion of the hepatocellular carcinomas examined (22). We also observed that the levels of phosphorylated MEK1/2 do not correlate with the levels of phosphorylated ERK1/2. This raises the possibility that in hepatocellular carcinoma, ERK1/2 may not only be the substrate of MEK1/2 but also of other kinase(s). The high expression of phosphorylated MEK1/2 seen in the 1318 series suggests that these lines are useful models for testing MEK inhibitors.

In the present study, we observed that blocking EGFR had minimal or no effects on xenograft growth. This is in contrast to a previous report (15) showing that ZD1839 inhibited growth of the implanted tumor and intrahepatic metastasis by 50%. The reason for this discrepancy remains unknown.

It has been reported that serum estradiol levels are elevated in patients with hepatocellular carcinoma and in those with liver cirrhosis (16, 33). Cirrhotic patients with hepatocellular carcinoma have significantly lower plasma concentrations of testosterone, dihydrotestosterone, and dehydroepiandrosterone than patients with cirrhosis alone (16). Furthermore, low levels of testosterone in male hepatocellular carcinoma patients and high levels of progesterone in cirrhosis patients have been observed (33). The results obtained from our hepatocellular carcinoma xenografts indicate that castration and steroid hormones, when supplemented as single agents to castrated...
mice, have no influence on the growth behavior of androgen receptor/estrogen receptor/progesterone receptor–positive hepatocellular carcinoma xenografts. Therefore, it remains to be determined whether the changes in circulating steroid hormones in the hepatocellular carcinoma group reported by previous studies (16, 33) are crucial for the development and progression of hepatocellular carcinoma.

Although a significant decrease in hepatocellular carcinoma incidence in C3H/Sy mice after long-term administration of vitamin D analogue EB1089 has been reported (19), we observed EB1089 to have no effects on hepatocellular carcinoma xenograft growth (Table 2). The reasons for the differences in tumor response between the two experimental systems are unknown. It has been proposed in a report of a phase II clinical trial of hepatocellular carcinoma that EB1089 may have an effect in the treatment of hepatocellular carcinoma, especially in early disease, when a prolonged treatment is administered (34). It is possible that the duration of administration of EB1089 plays a role in determining the response.

We report that among the several common chemotherapeutic drugs tested in our hepatocellular carcinoma xenografts, only doxorubicin exerts significant growth suppression. These observations are consistent with a prospective Hong Kong clinical trial that shows a small survival benefit with doxorubicin over placebo (10.6 versus 7.5 weeks; reviewed in ref. 18). In the present study, doxorubicin, given at a dose of 1 mg/kg by i.v. injection every 3 days for 3 weeks, causes a significant loss of body weight. This could be due to its known toxicity. Whereas oxaliplatin or cisplatin had no effects on tumor growth, administration of 5-FU results in mild growth suppression. This is in agreement with the results from various clinical trials (reviewed in refs. 18, 35). The mechanisms by which doxorubicin and 5-FU inhibit the growth of hepatocellular carcinoma xenografts remain to be determined. In the present study, doxorubicin exerts its antitumor activity, at least in part, by an up-regulation of p53 and p21Cip1/Waf1. In contrast to a previous study (36), our in vivo study shows an association between doxorubicin-induced growth suppression and up-regulation of p21Cip1/Waf1. In addition to inhibition of cell proliferation, doxorubicin also increases cleaved caspase-3 labeling index, suggesting that apoptosis also takes place following doxorubicin treatment. Recently, many targeted drugs have been developed for the treatment of cancers, including MEK, Raf, and vascular endothelial growth factor receptor 2 inhibitors (reviewed in refs. 7, 37). With the availability of hepatocellular carcinoma xenografts, combinations of low dose of doxorubicin or 5-FU with MEK or Raf or vascular endothelial growth factor receptor 2 inhibitors can be tested. These combinations may produce better results in hepatocellular carcinoma with less toxicity than monotherapy.

Similar to our previous study (14), significant growth inhibition is obtained when SarCNU is administered at the dose of 60 mg/kg for 4 days as determined by the final tumor weight (Fig. 4C). Body weight loss associated with toxicity is observed with high doses of SarCNU (Fig. 4A). The mechanisms responsible for the SarCNU inhibition of hepatocellular carcinoma xenografts are not currently known. In addition to elevation of p53 and p21Cip1/Waf1, phosphorylation of cdc-2 at Tyr15 following SarCNU may play a key role in SarCNU-induced growth inhibition. This hypothesis is...
supported by our recent studies in hepatoma (13) and prostate cancer cells (14). In these studies, we showed that inactivation of cdc-2 following SarCNU treatment is responsible for its antiproliferation as determined by Ki-67 and cleaved caspase-3 labeling index. Our data suggest a potential use of SarCNU in the treatment of hepatocellular carcinoma.

In summary, we have established and characterized seven lines of hepatocellular carcinoma xenografts. By means of these xenografts, we have shown that the majority of chemotherapeutic drugs currently used in the treatment of hepato-cellular carcinoma has little or no antineoplastic activity in vivo. With the availability of hepatocellular carcinoma xenografts, novel compounds and/or various combinations of chemotherapeutic drugs can be tested and compared before clinical trials.

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