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# Green Tea Polyphenols Mediated Apoptosis in Intestinal Epithelial Cells by a Fadd-Dependent Pathway

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## ABSTRACT

*Colorectal cancer is the most common malignant complication in patients with chronic inflammatory bowel disease (IBD). In addition, these patients are at risk for developing painful complications during chemotherapy due to cytotoxic effects of drugs currently in use. Past studies have suggested a protective effect of tea consumption on gastrointestinal (GI) malignancies. Green tea polyphenols (GrTP) inhibited carcinogen-induced GI tumors in rodents and induced apoptosis in various carcinoma cell lines. We hypothesized that GrTP and its polyphenolic compounds regulate apoptosis in the intestinal epithelia. In this study, the effects of GrTP and its polyphenolics on apoptosis was evaluated in intestinal epithelial, IEC-6, cells grown to 85% confluency. GrTP (400-800 mg/ml) induced DNA fragmentation in a dose dependent fashion. Higher concentrations (> 800 mg/ml) induced a mixed apoptosis and cytolysis. Epithelial cells exposed to GrTP and a major polyphenol, EGCG, but not EGC or EC, increased caspase activities in a time and dose dependent manner. The caspase inhibitors rescued cells from GrTP and EGCG-induced cell death. Concomitantly, GrTP resulted in activation of fatty acid synthase (Fas)-associated protein with death domain (FADD) and recruitment to Fas/CD95 domain 30 minutes following treatment. While GrTP also blocked NF-κB activation, an NF-κB inhibitor (MG132) only promoted cytolysis. In conclusion, these data demonstrated GrTP and EGCG induced apoptosis in intestinal epithelia mediated by caspase-8 through a FADD dependent pathway. Future investigation may warrant preventive as well as therapeutic strategies for GrTP in GI malignancy.*

***Keywords*: *Intestinal Epithelial Cells, Green Tea Polyphenols, EGCG, Apoptosis***

## 1. Introduction

Cancer is known as one of the most prevalent causes of mortality, and colorectal cancer is the most common malignant complication in patients with chronic idiopathic inflammatory bowel disease (IBD) [1]. It is estimated that one in six IBD patients develop colorectal cancer [2], suggesting a profound relationship between the chronic inflammation of IBD and susceptibility to developing colorectal cancer. In addition, these patients are at risk for developing severe gastrointestinal (GI) complications, including diarrhea, and abdominal pain during chemotherapy due to the cytotoxic effects of current anticancer drugs.

In a normal individual, the number of intestinal epithelial cells (IEC) on the villous and crypt are tightly regulated. IEC are generated from stem cells in the crypt and migrate to the villous tip in about 2-3 days, then "sloughed" [3-4], and replaced by new epithelial cells so that the villous height remains constant. This process is mainly regulated through apoptotic pathways.

Evidence indicates that defective apoptosis, impaired intestinal epithelial barrier function, activation of macrophages, and increased production of macrophage-derived cytokines (*i.e.*, TNFα) are critical in the inflammatory cascade leading to IBD (5-6). An additional trigger of this process is the host response to the commensal gut microbiota, through innate-defense mechanisms [5-7]. In Crohn's patients the lamina propria T lymphocytes (LPL) of the intestinal mucosa are chronically activated (6). Defective apoptosis of activated LPL is considered a key pathognomonic mechanism. In fact, increased expression of anti-apoptotic molecules is reported in LPL from these patients [8].

Apoptosis is a gene-directed cellular self-destruction and is recognized by its distinctive morphology, including the fragmentation of DNA into oligomers (180-200 bp) and DNA-ladder formation resulting in later stage activation of endonucleases. Initiation of apoptosis involves extrinsic and intrinsic pathways requiring enzymatic activities of cysteine-aspartate specific proteases "caspases cascades" [9]. The fatty acid synthase (Fas) associated death domain protein (FADD) dependent pathway initiates apoptosis via its death domain which binds to sequence motifs within the prodomain of FLICE/MACH/Caspase-8 and thereby recruits this apical regulator protease that initiates activation of downstream caspases (e.g., CPP32/caspase-3).

The balance between generated proapoptotic and anti-apoptotic proteins determines in part, how cells react to apoptotic signaling. Cancerous cells are generally more resistant to apoptotic pathways [9]. Specifically, resistance to cell death (anoikis), triggered by the loss of anchorage to the substratum, has been reported as an essential prerequisite in the proliferation and diffusion of colorectal cancer cells [10].

Various studies suggest a protective effect of tea consumption on GI health and in malignancies. Green tea polyphenols (GrTP) from *Camellia sinensis* have been reported to inhibit inflammatory responses [11-15] and carcinogen-induced GI tumors in rodent models [16-17]. In addition, GrTP have been shown to inhibit cell growth and are capable of inducing apoptosis in several carcinoma cell lines [18-21]. These polyphenolic compounds are constituted mainly from (-)-epigallocatechin gallate (EGCG) among 4 polyphenolic catechins, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC) [11].

However, the protective mechanism(s) of action of GrTP for intestinal cell biology and the pathway(s) involved in green tea-induced apoptosis in these tissues remains unclear. In this study we investigated the effect of GrTP and its major polyphenol, EGCG, on apoptosis and caspase activation in intestinal epithelial cells.

## 2. Materials and Methods

**Materials:** Extracted green tea polyphenols (HPLC graded GrTP; > 95% polyphenols) were purchased from LKT Laboratories, Inc (St. Paul, MN). High-performance liquid chromatography analysis of the green tea extracts revealed the percentage composition of the four polyphenols of interest: 35% EC, 4% EGC, 15% EGC, and 38% EGCG. Individual polyphenolic constituent, EC, EGC, EGC, and EGCG (> 98% pure) were purchased from Sigma Chemical (St. Louis, MO). Disposable culture plasticware was purchased from Corning (Corning, NY). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA) and Bio-Whittaker (Walkersville, MD). Material for electrophoresis was purchased from BioRad (Hercules, CA) and the remaining biochemicals were obtained from Sigma Chemical (St. Louis, MO).

**Cell culture and experimental design:** The fetal rat intestinal epithelial cell line, IEC-6, was initially obtained from the American Type Culture Collection (ATCC, Rockville MD). These non-tumorigenic cells are derived originally from intestinal crypt epithelia, and retain their distinct morphological and immunological characteristics through serial culture.

The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Bio-Whittaker, Walkersville, MD) supplemented with 10% (v/v) endotoxin-free fetal calf serum, 2 mM glutamine, and 100 U/ml penicillin/100 μg/ml streptomycin, at 37°C in an atmosphere of 10% $CO_2$ and 95% relative humidity. The media was changed every 3 days. Passage consisted of incubating cells in sterile trypsin/EDTA followed by resuspending the cells in fresh medium. The cells were studied between passages 15 and 20. Approximately $1 \times 10^5$ cells per well were plated in 6-well plates (Corning, Corning, NY) with 1 ml of media per well or seeded in 100-mm petri dishes with 7 ml of media per petri dish. Cells were used for experiments when they became confluent (85%), usually within 3 to 5 days after plating.

**Cell viability assays:** Cell viability was assessed using a formazan-based assay [22]. Following treatment of cells in 96 wells at $1 \times 10^4$, the incubation medium was removed and replaced with 80 μl of fresh medium. Twenty μl of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, Sigma, St. Louis) solution was added (5 mg/ml stock), and cells were incubated at 37°C for 3 h. Medium was then removed, and 150 μl of DMSO was added and allowed to incubate for 10 min. The degree of formazan production was measured by spectrophotometer at 490 nm. The average absorbance reading was taken from each time point of each plate. Cell survival curves were constructed by plotting cell survival against the various treatments. In addition cells were further examined microscopically using a trypan blue exclusion assay.

**DNA fragmentation:** DNA from treated cells was extracted and assayed as follow. Cells were collected and lysed with 1.0 ml of lysis buffer [100 mm NaCl, 10 mm Tris (pH 8.0), 25 mm ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate (SDS), 200 μg/ml of DNase free proteinase K (Sigma)] and treated with DNAse free RNase (40 μg/ml) in the presence of 0.1% SDS for 1 h at 37°C. DNA was reextracted with phenol/chloroform, precipitated with 100% ethanol and resuspended in sterile-distilled water. Ten μg of extracted DNA were loaded into each well and electrophoresed in

1.5% agarose gel (BioRad, Hercules, CA). Gels were stained with ethidium bromide and visualized by ultraviolet fluorescence and photographed with a Polaroid camera system.

**Caspase activity assay:** Caspase activity was detected by a Quantizyme Assay System (Biomol, Plymouth, PA). The enzyme activities and cleavage of caspase 3 and caspase 8-9 were determined colorimetrically using Ac-DEVD-pNa (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline) and Ac-ITED-pNA as substrates respectively and absorbance monitored at 405 nm. The specific caspase-3 (z-DEVD 100 µmol) and caspase-8 and -9 inhibitors (Z-ITETD) were added to the cells immediately before stimulation with GrTP and EGCG. The cells were washed with cold phosphate-buffered saline (PBS) and adherent cells were pelleted by centrifugation at 400 g for 5 min at 4°C. The cell pellet was washed with ice-cold PBS and resuspended in 100 mmol/l HEPES buffer (pH 7.4) containing protease inhibitors (5 µg/ml aprotinin, 10 µg/ml leupeptin, and 0.5 mmol/l phenyl methyl sulfonyl fluoride (PMSF). The cell suspension was lysed by 3 freeze/thaw cycles, and the cytosolic fraction was obtained by centrifugation at 12,000 g for 20 min at 4°C. Protein concentration was determined using the BCA assay (Sigma, St Louis MO). Cytosolic protein (50 to 100 µg) was combined with 100 µmol/L (2 µl) of the synthetic substrate for caspases-3, -8, and -9 in a total volume of 100 µl of HEPES (100 mmol/l, pH 7.4) containing protease inhibitors. The reaction was conducted for 1 h at 37°C. Cytosolic caspase activities were assayed by measuring the increased absorbance and caspase activity.

**Fas/FADD complex analysis:** The Fas/CD95 and its associated proteins were immunoprecipitated from cell lysates using mouse anti-Fas antibody and protein G plus agarose. After extensive washes the immuno-complex was denatured, and resolved by 15% SDS-PAGE performed in mini slab gel unit (BioRad, Hercules CA). The proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in PBS-Tween buffer [80 mM $Na_2HPO_4$, 10 mM NaCl, and 0.05% Tween-20 (pH 7.5)] containing 10% nonfat milk. To identify FADD the membrane was incubated for 2 h with specific anti-FADD antibody. To ensure equal loading of samples the membrane was re-probed with mouse anti-Fas antibody. Anti-FADD and anti-Fas antibodies were visualized by an enhanced chemiluminescence blotting kit (ECL, Amersham, Bioscience, Pittsburgh, PA). Band intensity was quantified by the absolute integrated optical intensity.

**Statistical analysis:** Results are expressed as mean ± SD unless otherwise stated. Data were evaluated using ANOVA, followed by appropriate *post hoc* test using GraphPad Instat version 3 for Windows (GraphPad Software, San Diego, CA). Statistical significance was set at $p < 0.05$.

## 3. Results

Intestinal cells were treated with different concentrations of GrTP (100-400 µg/ml) or EGCG and EGC (10-500 µM) for a period of 24 h. Cell viability and death were determined with trypan blue exclusion and confirmed with MTT assay. While lower doses of GrTP had no significant effect on the viable cells, higher concentrations caused cell death in a dose dependent manner. GrTP induced (43 ± 0.5) cell death at a high dose of 400 µg/ml (**Figure 1(a)**) which correlated with DNA fragmentation and ladder formation in these cells (**Figure 1(b)**). There was no significant differences ($p > 0.05$) detected between untreated control cells and those treated with lower doses of GrTP (100-200 µg/ml). GrTP at higher concentrations (400-800 µg/ml) induced DNA fragmentation in a dose responsive fashion, although, higher concentrations (> 800 µg/ml) of GrTP promoted a mixed portrait of apoptosis and cytolysis (data not shown). Accordingly,

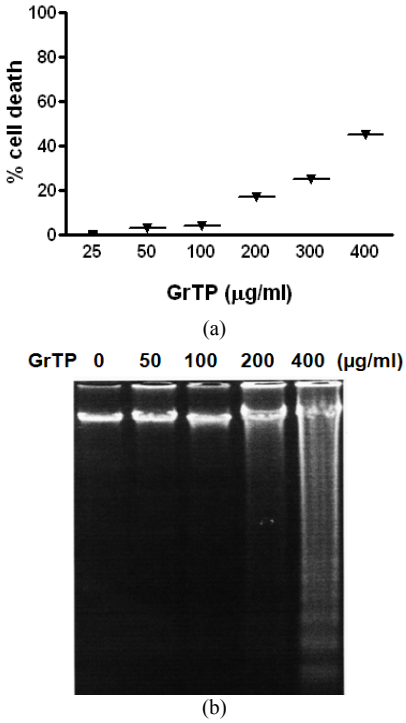


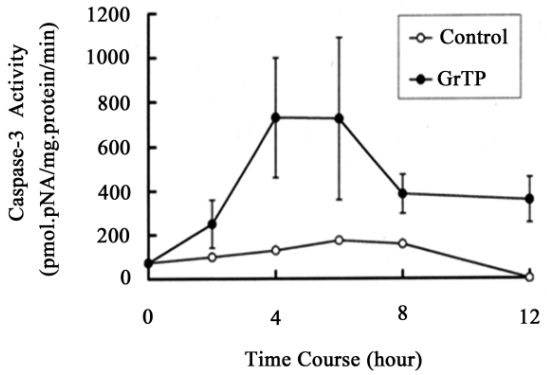

**Figure 1. Intestinal Epithelial Cells (IEC-6) were treated with different concentrations of Green tea polyphenols (GrTP) for 24 h. (a) GrTP induced cell death in IEC-6 cells. The data is expressed as mean ± SD, n = 3; (b) GrTP induced DNA fragmentation in IEC-6. Cells were treated with indicated doses of GrTP for 24 h.**

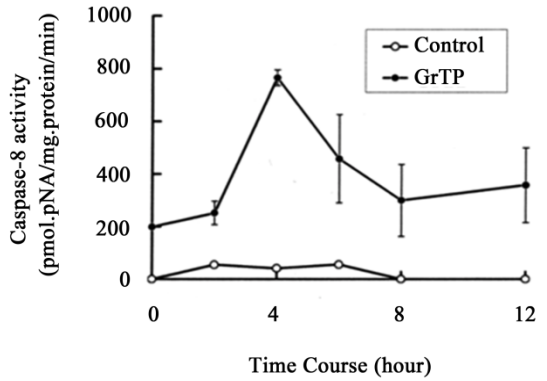
GrTP upregulated caspase-3 activity that was detected within 2 h ($p < 0.01$) and increased after 4 h ($p < 0.001$) of intestinal epithelial cell' exposure (**Figure 2(a)**). GrTP also increased caspase-8 activity in a similar fashion with a sharp peek after 4 h ($p < 0.001$) of exposure (**Figure 2(b)**). GrTP (**Figure 3(a)**) and EGCG (data not shown) blocked TNFα induced IL-8 release from intestinal epithelial cells in a dose dependent fashion ($p < 0.001$). TNFα by itself (25 ng/ml) did not induce DNA fragmentation in intestinal epithelial cells, nor did it synergize with GrTP in this outcome (**Figure 3(b)**).

Although, GrTP decreased NF-κB activation in intestinal epithelial cells, the NF-κB inhibitor (MG132) promoted only cytolysis and with no induction of apoptotic events (**Figure 4**). In contrast, treatment of cells with specific translational inhibitors, staurosporine and actinomycin D (50 ng/ml), provoked DNA fragmentation of intestinal epithelia is similar to high doses of GrTP (400 µg/ml) (**Figure 4**). In addition, epithelial cells were pretreated with actinomycin D, staurosporine or sham control (saline) for 30 min and then TNFα (25 ng/ml) or PBS was added for 15 h. TNFα treatment alone or in combination with these translational inhibitors did not provoke nor synergize in eliciting DNA laddering.

Concomitantly, GrTP activated FADD and recruited the Fas/CD95 domain in a time dependent manner evidenced 30 minutes after cell treatment. This increased at

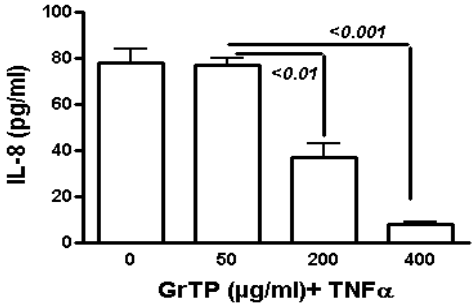



(a)

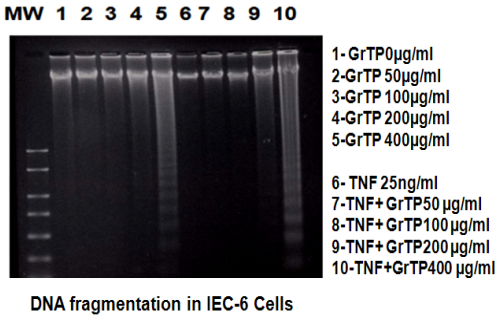



(b)

**Figure 2. (a) Activation of caspase-3 and (b) caspase-8. IEC-6 cells were treated with 400 µg/ml of GrTP for the indicated time. Caspase activities were significantly increased following exposure of cells to GrTP after 4 h ($p < 0.001$) into the incubation. Data is expressed as mean ± SD, (n = 3).**




(a)




(b)

**Figure 3. GrTP prevented NF-κB activation and IL-8 release mediated by TNFα stimulation of IEC-6 cells. (a) shows a dose dependent response of GrTP treatment; (b) demonstrates inhibition of DNA degradation.**

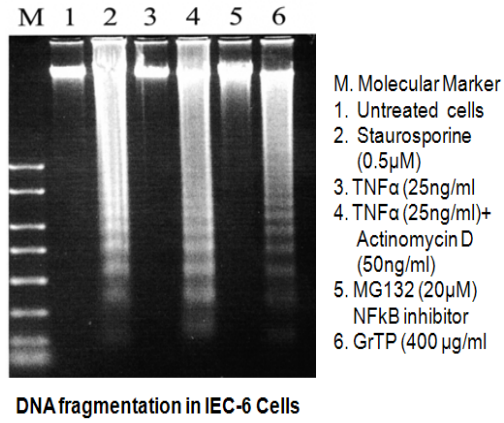



**Figure 4. Induction of DNA fragmentation in IEC-6 cells. Cells were treated with indicated stimuli for 24 h. (M) denotes molecular weight marker, (1) Untreated cells; (2) Staurosprine (0.5 µM); (3) TNFα (25 ng/ml); (4) TNFα (25 ng/ml) + Actinomycin D (50 ng/ml); (5) MG132 (20 µM); (6) GrTP (400 µg/ml).**

1 h, as shown by Western blot analysis (**Figure 5**) demonstrating activation of death pathways through a FADD dependent pathway.

We measured apoptosis induced by different concentrations of various catechins in GrTP. The major catechin component of GrTP is EGCG (approx. 40%). Amongst polyphenols, EGCG at 100 μM caused activation of caspase-3 and caspase-8 in a time dependent manner which sharply increased after 8 h of treatment (**Figure 6**). Minimal caspase activation was induced by other polyphenolic constituents (EC or EGC data not shown). Lower doses of EGCG (10-50 μM) did not promote caspase activation. The caspase activity was prevented by specific (caspase-3) and general (caspase-8 and -9) caspase inhibitors leading to cell survival rescued after treatment which mimicked untreated normal intestinal epithelia.

## 4. Discussion

We investigated the efficacy of GrTP and its polyphenolic constituents to regulate apoptosis in fetal rat intestinal epithelial cells. While GrTP and EGCG in lower doses had no significant effect on cell viability, higher concentrations provoked apoptosis in a dose and time dependent manner which correlated with caspase (3,8 and 9) activation.

Under normal circumstances, the gut immune system is in a constant balance between inflammatory and apoptotic states, as epithelial cells and activated T lymphocytes are rapidly and efficiently eliminated via apoptosis. However, IBD patients develop a dysfunction in apoptosis [23-25], that can contribute an increased incidence of colorectal carcinoma, and chemotherapy cytotoxicity of the gut epithelia. Apoptosis can be initiated from cell cycle activation [26-27], cytokine production [28], infective agents [29], or as an adverse response to drugs [30-31]. Based on the agonist or initiating milieu, apoptosis is accompanied by the activation of various cells

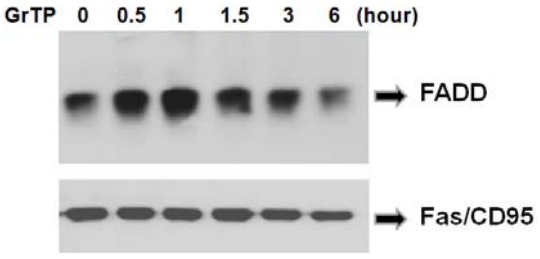



**Figure 5. GrTP induced recruitment of FADD to Fas/CD95. IEC-6 cells were treated with 400 μg/ml of GTP for the indicated time. The Fas/CD95 and its associated proteins were immuno-precipetated using anti-Fas antibody. The presence of FADD was identified by Western immunoblotting. Equal loading of samples was verified by re-probing the membrane with anti-Fas antibody.**

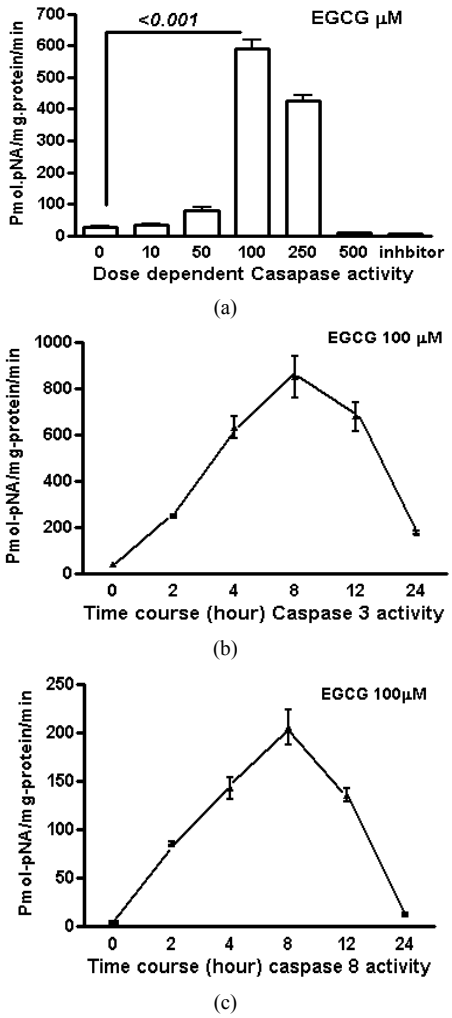



**Figure 6. EGCG induces caspase activation in IEC-6 cells. To provide dose and time dependent response relationship, cells were treated with different concentrations of EGCG. (a) or 100 μM/ml of EGCG for the indicated time, and caspase' activity was measured (b-c). EGCG at 10-50 μM had no caspase activity ($p > 0.05$). Caspase inhibitor rescued the cells from EGCG apoptotic effects of EGCG activated -3; (b) and to a lesser extend caspase-8; (c) in treated cells with a time depended response. Data expressed as mean ± SD (n = 3). Also, caspase inhibitors rescued the cells from EGCG apoptotic effects.**

death pathways such as caspases, altered gene expression, mitochondrial dysfunction and consumption of ATP with DNA repair [32]. Apoptosis is crucial in maintenance of intestinal epithelial integrity and regulated by numerous factors including NF-κB activity [33]. It is speculated that excessive secretion of cytokines including IL-6 in the gut of Crohn's disease patients, upregulates expression of antiapoptotic molecules and impairs mucosal homeostasis [34]. The transcription inhibitor and chemo-

therapeutic agent, actinomycin D, elicits apoptosis in cell types, by anchoring into (purine–pyrimidine) DNA-base pairs via intercalation and inhibiting mRNA biosynthesis [35]. Early studies demonstrated that actinomycin D enhanced the rate of apoptosis in the intestinal epithelial crypts in mice [36] and the apoptotic bodies were either extruded into the lumen or phagocytized and degraded by adjacent cells with essentially different ultrastructure than necrosis induced in the center of tumors [36]. The distinctive morphology of apoptotic cells included, chromatin condensation, cytoplasmic shrinkage, and membrane-bound apoptotic bodies and genomic breakdown. In this study high dose GrTP provoked fragmentation of the intestinal epithelial genome into oligomers similar to actinomycin D and staurosporine.

TNFα a pleiotropic cytokine is involved in regulation of proinflammatory gene expression [37-38], cytotoxicity, apoptosis and pathogenesis in IBD patients [5,39] and in IBD-models [13,40]. Therapeutic modalities using monoclonal antibodies to neutralizing or reducing TNFα result in reduction in mucosal inflammatory response [5,41]. GrTP significantly lowered TNFα production, cyclooxygenases and B-cell lymphoma (BCL)-2 upregulation, and restored glutathione resources, which protected against acetaminophen induced hepatotoxicity [12]. TNFα treatment alone or combined with specific translational inhibitors actinomycin D and staurosporine did not provoke nor synergize in eliciting DNA laddering in these epithelia cells. The present data also extends these findings by confirming that, TNFα stimulation, by itself, is not a major factor inducing apoptosis in intestinal epithelia [42-43]. Also, caspase activation is required in these cells for the progression of apoptosis [44-45]. EGCG treatment of cells resulted in a significant increase in protein expression and activation of caspases and induced proteolytic cleavage of NF-κB/p65 subunit, leading to the loss of transactivation domains, and apoptosis [46]. In addition, GrTP and EGCG-mediated apoptosis was significantly blocked by caspase inhibitors.

Murine intestinal epithelial cells (IEC-18) exposed to oxidants, peroxynitrite and $H_2O_2$, and pretreated with low dose green tea were protected against necrotic cell death [31]. Dysregulation of intracellular redox balance with depletion of antioxidant reserves is associated with the activation of transcription factors like NF-κB, and genes associated with apoptosis (TNFα), proliferation and inflammation [12,28,33,47]. Indeed, dietary antioxidants including green tea [46,48] inhibited gene expression associated with inflammation, and immune activation, and protected the gut from pro-apoptotic oxidant stress at levels distinct from the scavenging of the oxidant signal alone. Taken together, these studies demonstrate that GrTP and EGCG-mediated activation of caspases is critical, at least in part, for inhibition of NF-κB and subsequent apoptosis.

The 2 principal signaling pathways that mediate apoptosis are associated with the secondary generation of ROS [49], and mitochondrial dysfunction in cells, which trigger mitochondria-dependent apoptosis (intrinsic pathway) [10]. Alternatively, over-expression of antioxidants such as intracellular superoxide dismutase reduced induction of cell death [50]. The extrinsic pathway (Fas/FasL) initiates recruitment of FADD to the death receptors (TNF receptor, Fas/CD95) and causes autocatalytic activation of caspases leading to apoptosis [10]. Colonic cancer tissues show alterations in the CD95 (Fas/FasL) as tumors progress from local to metastatic disease. Fas antigen inhibits apoptosis by increasing Fas-mediated proliferation and cell survival in local colonic tumor growth, and coexpression of FasL may be involved in metastatic nature of these neoplastic tumors [10].

GrTP have been shown to inhibit carcinogenesis and tumorigenesis on different organs in models. Green tea is speculated to modulate the signaling pathways leading to transformation, and apoptosis of preneoplastic and neoplastic cells, as well as inhibition of tumor invasion and angiogenesis [16]. For instance, EGCG exerts grow- th inhibitory effects on a number of human tumor cells [20-21,49,51] including colorectal cancer (Caco-2) cells [18]. The anti-proliferative effect of EGCG in Caco-2 cells is possibly modulated by *c-fos* and *c-myc* gene expression [18]. Based on these findings, EGCG may be a candidate for consideration as treatment option for several human carcinomas.

GrTP induced DNA fragmentation in IEC-6 cells in a dose and time dependent manner. At doses above 200 μg/ml GrTP activated caspase-3 and caspase-8 in these cells that peaked 4 h following exposure consistent with increased recruitment of FADD to Fas/CD95 and formation of Fas/FADD complex. Overall GrTP and EGCG may be useful in the chemoprevention of carcinomas with overexpression of Fas signaling. Tumor cells with deleted caspase-3 gene treated with GrTP did not undergo apoptosis, suggesting GrTP-induce apoptosis is a mitochondria-targeted and, caspase-3 executed mechanism in the tumor cells [52]. In addition Fas/Fas ligand deletion was associated with increased levels of IFNγ, TNFα and Fas expression and significant reduction in mouse intestinal epithelial damage and apoptosis [53].

Our previous investigations demonstrated that GrTP and EGCG in low concentrations blocked NF-κB activation and translocation in intestinal epithelial cells [11]. The NF-κB inhibitor, MG132, promoted only cytolysis with no induction of apoptotic events, indicating that GrTP may act by different mechanism(s) than solely inhibiting NF-κB activation. At low concentrations GrTP

acts as an antioxidant and inhibits NF-kB activation [11-14], while in higher doses it provoked apoptosis, as documented in this study. The anti-tumor efficacy of several chemotherapeutic agents is correlated with their ability to induce apoptosis [54,55]. Some of these regimens are partially effective in tumor cells being particularly resistant to radiotherapy and/or chemotherapy [55]. These data support that apoptosis induced by GrTP is caspase-dependent and involves both extrinsic and intrinsic pathways. EGCG has been reported (800 mg/day) to be safe with minor side effect in a preclinical trial [56]. Therefore, a safer and effective apoptotic agent represents an attractive approach for the development of potential anticancer agents and, GrTP may serve as a candidate for future translational cancer studies and preventive or therapeutic modality for GI malignancy.

## 5. Acknowledgements

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## Abbreviations

**Caspases**, Cysteine-aspartate specific proteases; **DISC**, death inducing signalling complex; **EC**, (-)-epicatechin; **EG**, (-)-epigallocatechin; **EGC**, (-)-epigallocatechin; **ECG**, (-)-epicatechin gallate; **EGCG**, (-)-epigallocatechin gallate; **Fas**, Fatty acid snthase; **FasL**, Fas Ligand; **FADD**, Fas associated death domain; **FLIP**, FADD-like inhibitory protein; **GI**, gastrointestinal; **GrTP**, Green tea polyphenols; **IL**, interleukin; **IBD**, Inflammatory bowel disease; **IEC**, Intestinal epithelial cells; **LPL**, lamina propria T lymphocytes; **NF-κB**, Transcription nuclear factor κB; **PBL**, peripheral blood lymphocytes; **ROS**, reactive oxygen species; **TNFα**, Tumor necrosis factor.

 

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# Genetic Polymorphisms of Hepatic ABC-Transporter in Patients with Hepatocellular Carcinoma

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## ABSTRACT

*We examined whether genetic polymorphisms of efflux transporters in hepatocytes are associated with susceptibility to develop hepatocellular carcinoma* (*HCC*). *Genetic polymorphisms of drug transporters expressed in hepatocytes were analyzed using DNA samples from hepatitis C virus* (*HCV*)*-seropositive cirrhotic patients with HCC* (*n* = 58), *and allele and haplotype frequencies were compared with those in healthy subjects* (*n* = 61). *To search for single nucleotide polymorphisms* (*SNPs*) *in HCC susceptibility genes,* 34 *SNPs in* 6 *efflux transporters* [*MDR*1 (*ABCB*1), *ABCC*1, *ABCC*2, *ABCC*3, *ABCG*2 *and ABCB*11] *were determined. No significant association was observed for any single SNP; however, some haplotypes in ABCC*1 *and ABCB*11 *were associated with HCC. Furthermore, three combinations of SNPs* (3435*C* > *T in ABCB*1 *and* 825*T* > *C in ABCC*1), (3435*C* > *T in ABCB*1 *and* -15281_-15278*CTCT* > *delete in ABCB*11), *and* (825*T* > *C in ABCC*1 *and* -15281_-15278*CTCT* > *delete in ABCB*11) *were significantly associated with HCC. The present study suggests that genetic variations of ABC transporters such as ABCB*1, *ABCB*11, *and ABCC*1 *are associated with susceptibility to develop HCC, implying that aberrant hepatic clearance of toxic substances may increase the risk of hepatocarcinogenesis. Further studies of how these polymorphisms are associated with phenotypic differences are warranted.*

***Keywords*: *Genetic Polymorphism, Drug Transporter, Hepatitis C Virus, Hepatocellular Carcinoma***

## 1. Introduction

Hepatocellular carcinoma (HCC) is a frequent complication in advanced chronic liver disease, and is a major cause of death worldwide. In Japan, the high prevalence of HCC has largely been attributed to chronic infection by hepatitis virus, especially hepatitis C virus (HCV) [1-3]. HCV infection with persistent inflammation leads to sequential progression from acute to chronic hepatitis, cirrhosis, and eventually HCC in some cases. Since the development of HCC is a multistep process, numerous host factors (including gender, age, ethnicity, and stage of liver disease), viral factors (including viral genotype and levels), and environmental factors (including carcinogens, medications, and food) have been reported to be involved [4]. Further, oxidative products from endogenous and exogenous substances may accumulate in hepatocytes, possibly leading to hepatocarcinogenesis. One important protective function of the liver is the biliary and sinusoidal clearance of such endogenous and exogenous mutagenic/carcinogenic substances, preventing their accumulation in hepatocytes. This function is maintained by a drug efflux transporter system that comprises mainly the ABC-transporter proteins [5,6]. Therefore, mutation(s) of these proteins may impair the protective system against accumulation of hazardous compounds that may lead to HCC.

Recently, various drug transporters expressed in hepatocellular membranes have been studied in terms of vectorial direction, substrates, and the pharmacokinetics (PK)/pharmacodynamics (PD) of clinically relevant drugs. Among the efflux transporters, ABC transporters such as multidrug resistance 1 (MDR1, p-glycoprotein) are important in the detoxification of xenobiotics. For example, p-glycoprotein acts as an efflux pump for the carcinogen benzo[*a*]pyrene in human breast cancer cells [7,8].

An increasing number of single nucleotide polymorphisms (SNPs) in drug transporter genes are being iden-

 

tified in humans [8], and may account for many of the significant individual differences in susceptibility to exogenous and endogenous mutagenic and carcinogenic insults. An example is 3972C > T mutation in *ABCC*2 (MRP2 gene); the risk of intrahepatic cholestasis of pregnancy for 3972T/T carriers has been reported to be 4-fold higher in comparison with 3972C/C carriers [9]. With regard the mutation 421C > A in *ABCG*2 (BCRP gene), 421C > A was associated with risks of diffuse large B-cell lymphoma and renal cell carcinoma [10,11]. Thus, the role of genetic polymorphisms of drug transporters in the development of HCC in patients infected with HCV is of interest. In this study, we selected 6 candidate genes encoding efflux transporters, which are involved in the hepatobiliary transport of endogenous and exogenous compounds in humans [8], and compared the allelic and haplotypic frequencies based on 34 SNPs between HCV-positive HCC patients and healthy subjects.

## 2. Materials and Methods

### 2.1. Subjects and DNA Samples

Fifty-eight Japanese cirrhotic patients with HCC were studied. Cirrhosis and HCC were diagnosed by liver biopsy or computed tomography and ultrasonography. Patient characteristics are summarized in **Table 1**. Peripheral blood was collected after obtaining written informed consent. The samples were numbered, unlinked, and test- ed anonymously. DNA was extracted by the standard protocol. The use of patient blood samples for this study had been approved by Jikei University Ethics Committee. For comparison, genomic DNA was also obtained from 61 Japanese healthy volunteers (aged 20 to 40 years). Each subject was physically normal and had no antecedent history of significant medical illness or hypersensitivity to any drug. Their body mass indices ranged between 18 and 26 kg/m$^2$. Their health status was judged to be normal based on a physical examination, screening of blood chemistry, complete blood count, urinalysis, electrocardiogram, and chest X-ray conducted before the study. Written informed consent was obtained from all participants before the study.

### 2.2. Genotyping

Among various efflux transporters, we analyzed the following six ABC (ATP-binding cassette) transporters: multidrug resistance 1 (MDR1, p-glycoprotein; gene name, *ABCB*1); multidrug resistance-associated protein 1 (MRP1, *ABCC*1), 2 (MRP2/cMOAT, *ABCC*2) and 3 (MRP3, *ABCC*3); breast cancer resistance protein (BCRP, *ABCG*2); and bile salt export pump (BSEP, *ABCB*11). The polymorphisms examined are listed in **Table 2**. All occur at certain frequencies or are reported to have functional variability [12-18]. Bile salt export pump (BSEP) contributes to the efflux of bile acids or bile salts across the canalicular membrane in the liver [19,20]. Although defects of the gene encoding BSEP can lead to a hereditary disorder, progressive familial intrahepatic cholestasis II (PFIC II) [21,22], all coding exons, all exon-intron junctions, and some 5' upstream regions were screened because we hypothesized that certain polymorphisms may be associated with lower transport activity.

376C > T and 421C > A in *ABCG*2, and 3435C > T in *ABCB*1 were determined using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA) with TaqMan SNP genotyping assays. -1767G > A in *ABCC*3 were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The other polymorphisms were detected by PCR-single strand conformation polymorphism (PCR-SSCP). The accuracy of PCR-SSCP-based-genotyping

**Table 1. Patients' characteristics.**

| variable | unit | Mean ± SD | range |
|---|---|---|---|
| gender | M/F | 43/15 | |
| age | years old | 70 ± 7 | (53~87) |
| clinical stage (T.N.M)† | I/II/III/IVa/IVb | 11/29/15/2/1 | |
| Child-Pugh‡ | A/B/C | 48/7/3 | |
| AST | (IU/L) | 74 ± 31 | (26~157) |
| ALT | (IU/L) | 68 ± 62 | (11~459) |
| total protein | (g/dl) | 7.6 ± 0.8 | (5.5~9.5) |
| albumin | (g/dl) | 3.7 ± 0.5 | (2.5~4.6) |
| total bilirubin | (mg/dl) | 1.3 ± 2.2 | (0.3~16.5) |
| prothrombin index | (%) | 79 ± 12 | (44~100) |

Data are mean ± SD. Parenthesis indicates the range of data. †, TMN classification; ‡, Child-Pugh classification A, B, C; AST, asparate aminotransferase; ALT, alanine aminotransferase. Normal ranges (Central Chemical Laboratory, Daisan Hospital, The Jikei University School of Medicine, Tokyo): AST 5–28 IU/L; ALT 5–35 IU/L; total protein 6.8–8.3 g/dl; albumin 3.5–5.2 g/dl; total bilirubin 0.2–0.8 mg/dl; prothrombin index 80-100% (prothrombin index obtained by dividing the prothrombin time of the patient by the reference value of control subjects).

 

**Table 2. Polymorphisms examined in the present study.**

| gene | position | polymorphism | amino acid substitution | reference |
|---|---|---|---|---|
| *ABCB*1 | exon 21 | 2677G > T/A | 893Ala > Ser/Thr | [9-11] |
| | exon 26 | 3435C > T | 1145Ile (syn) | |
| *ABCC*1 | exon 8 | 825T > C | 275Val (syn) | [12] |
| | exon 9 | 1062T > C | 354Asn (syn) | |
| | intron 9 | 1218 + 8A > G | - | |
| | exon 13 | 1684T > C | 562Leu (syn) | |
| | exon 16 | 2007C > T | 669Pro (syn) | |
| | exon 17 | 2168G > A | Arg723Gln | |
| *ABCC*2 | promoter | -24C > T | - | [12] |
| | exon 10 | 1249G > A | 417Val > Ile | |
| | exon 18 | 2302C > T | 768Arg > Trp | |
| | | 2366C > T | 789Ser > Phe | |
| | exon 28 | 3972C > T | 1324Ile (syn) | |
| | exon 31 | 4348G > A | 1450Ala > Thr | |
| *ABCC*3 | promoter | -1767G > A | - | [13] |
| | exon 2 | 135G > T | 45Leu (syn) | |
| *ABCG*2 | exon 4 | 376C > T | 126Gln > stop | [14] |
| | exon 5 | 421C > A | 141Gln > Lys | |
| *ABCB*11 | promoter | -15281_-15278CTCT > delete | - | [15] |

was confirmed by direct sequencing using a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems). The primers and restriction enzymes used in this study are listed in **Table 3**. The primers designed for *ABCB*11 were based on the following sequences; GenBank acession numbers AC008177.3 (for promoter and exons 1-21) and AC069137.6 (for exons 22-28) (**Table 4**). The specificity of all primers was confirmed by direct sequencing.

### 2.3. Haplotype Analysis

For each gene, a haplotype analysis was performed with Arlequin ver. 3.11 (http://cmpg.unibe.ch/software/arlequin3/). The Arlequin software package estimates haplotype frequencies using the expectation-maximization (EM) algorithm. Only polymorphisms, except those in intronic regions, with a relative frequency > 0.05 were included in the analysis in either patients or healthy volunteers.

### 2.4. Analysis of Combinations of Polymorphisms in Different genes

In addition to the single gene testing, the influence of combinations of polymorphisms in different genes on HCC was assessed. In this analysis, we compared genotypic frequencies between subjects with and without variant types.

### 2.5. Statistical Analysis

All genotypes were tested as to whether they were distributed according to the Hardy-Weinberg equilibrium. The association between different genotypes and the presence of HCC was evaluated using the chi-square test. For all tests, a *P* value less than 0.05 was considered significant. The odds ratio and a 95% confidence interval (CI.) were also calculated.

## 3. Results

**Table 5** shows the polymorphisms of interest identified in the 6 genes, together with their allelic frequencies. In *ABCB*11, we identified 16 polymorphisms. Among them, 1331C > T (444Val > Ala) in exon 13 and 2594C > T (865Ala > Val) in exon 21 were non-synonymous variants. In the single gene testing, all 95% CI. values crossed 1.0, indicating no association between any SNPs and HCC.

Results of the haplotype assessment of HCC are shown in **Table 6**. Significant associations were observed in the *ABCC*1 ($p = 0.010$) and *ABCB*11 ($p = 0.028$) genes. In *ABCC*1, seven haplotype patterns were assessed on the basis of five SNPs (*i.e.*, 825T > C, 1062T > C, 1684T > C, 2007C > T, and 2168G > A). CCCCG and TTCCG were the most frequently observed haplotypes in the patient and control groups (CCCCG: 0.37 vs. 0.23; TTCCG: 0.25 vs. 0.36), respectively. The frequency of TTCCA was 0.06 in patients, while this haplotype was not observed in the control group. In *ABCB*11, eight haplotypes were assessed based on five polymorphisms (-15281_-15278 CTCT > delete, 108T > C, 807T > C, 1331T > C, and 3084A > G). DelTTCG was the most fre-

 

**Table 3. Primer sets for PCR-SSCP and PCR-RFLP determinations, and RELP conditions.**

| gene | polymorphism | forward primer | reverse primer |
|---|---|---|---|
| *ABCB*1 | 2677G > T/A | 5'-TACCCATCATCATTGCAATAGCAG-3' | 5'-TTTAGTTTGACTCACCTTCCC-3' |
| *ABCC*1 | 825T > C | 5'TGGTAGGGGGCTGCATCTCTG 3' | 5'CAAAGCCAAGGAGGGAAAATG 3' |
| | 1062T > C<br>1218 + 8 A > G | 5'CCCCCACGTGTCACAAGTCAT 3' | 5'CACGCTGGCCCCAGAGTAACC 3' |
| | 1684T > C | 5'ACTCGGGGCACAGCAGTCAGC 3' | 5'GAACCCCAAGGCCCCCTCTCG 3' |
| | 2007C > T | 5'TAGCCCGGCAGGCCTCATTCA 3' | 5'AGCTTTTCCTCAGACCACCAG 3' |
| | 2168G > A | 5'GTGGGCCAGCTGTTGTCTCGT 3' | 5'GCGGCAACAGCTGACTGATTC 3' |
| *ABCC*2 | -24C > T | 5'-CTGTTCCACTTTCTTTGATGA-3' | 5'-TCTTGTTGGTGACCACCCTAA-3' |
| | 1249G > A | 5'-AGCCACAAAGTAGCAGTGAGG-3' | 5'-TTACCCACAGAGAGCCACCTA-3' |
| | 2302C > T<br>2366C > T | 5'-GGAGTAGTGCTTAATATGAAT-3' | 5'-CCCACCCCACCTTTATATCTT-3' |
| | 3972C > T | 5'-AGGAGCTAACACATGGTTGCT-3' | 5'-GGGTTAAGCCATCCGTGTCAA-3' |
| | 4348G > A | 5'-AGGAGCTAACACATGGTTGCT-3' | 5'-GGGTTAAGCCATCCGTGTCAA-3' |
| *ABCC*3 | -1767G > A† | 5'-GCTTAGATATCACCCTGTCCA-3' | 5'-TCCACTCATCCACACATACCC-3' |
| | 135G > T | 5'-GGCTGCCACAGCACTAAACTG-3' | 5'-CCAGCCCCATCGGTAGGAGAT-3' |

†, PCR-RFLP was used for this analysis. The restriction enzyme was BsmA I (at 55°C for 2 hr) for RFLP. G allele was detected by 281 bp and A allele was detected by 241 bp plus 40 bp.

**Table 4. Primer sets for the PCR of *ABCB*11.**

| position | forward primer | reverse primer |
|---|---|---|
| promoter | 5'-TCCACTCCAGGTTCAGCTTC-3' | 5'-CTCTGCCTCAGGAATCTTACA-3' |
| exon 1 | 5'-CTCAATTTGCCTCTCGTTCCA-3' | 5'-GCATCCTGTAAGTTTCTATCC-3' |
| exon 2 | 5'-TTTCGTTTGGCTACTTTGATT-3' | 5'-ATACTTCTACCTACTGTTGCT-3' |
| exon 3 | 5'-ATTTCTTAATGACTGCGTTGC-3' | 5'-GGGGACATTTGAACCTAACCT-3' |
| exon 4 | 5'-GTGGTCTTTAAATCCTTATGT-3' | 5'-TGTGATGACTTTCCTTACAAA-3' |
| exon 5 | 5'-GATGATCTCTGAACCCTTTGT-3' | 5'-CAGCCAGTAAAATCCCCTCTA-3' |
| exon 6 | 5'-TGAACATTCTTTTCCCTCTTT-3' | 5'-ACATTGCATCTCATTGTAGTG-3' |
| exon 7 | 5'-CTGAATTACTTTCCCCCTTTT-3' | 5'-CATATTTGACAGTGTATTACT-3' |
| exon 8 | 5'-GTTTAAAAGGGAAAGACTGAG-3' | 5'-ATAATTATGTTGCTAACTGTA-3' |
| exon 9 | 5'-AATGACAGACTGACTTACCTA-3' | 5'-ACTCTGCTTAGCTCCCTCTTG-3' |
| exon 10 | 5'-ACTCAAGCATTTTGTCTTCAC-3' | 5'-ATGTCTCGGTCAATAAGTCCA-3' |
| exon 11 | 5'-GGAAGACCCAAATGATAGTAA-3' | 5'-GAATTAAGGGCCTTGCGATAG-3' |
| exon 12 | 5'-TCTTAGTTTGAGTTTACACTG-3' | 5'-ATTTACTATTCTGGGGAACAG-3' |
| exon 13 | 5'-GGGGCATACATAAACGCACAC-3' | 5'-ACTATGCATGCCAGGACAGTC-3' |
| exon 14 | 5'-CTGCCCATTGGTCAAGTATGA-3' | 5'-CTATGACCTCTTAGTTTCTCC-3' |

 

| | | |
|---|---|---|
| exon 15 | 5'-CATCAAATTCTTTTCCCTTCA-3' | 5'-GGACCTGTAAAATGGACTAAG-3' |
| exon 16 | 5'-TGAAATGATGCAAAGGTCAGT-3' | 5'-TAGAAAACCGTAAAGCACTAT-3' |
| exon 17 | 5'-TCTACTTGGATATGGTTCTGT-3' | 5'-ATTTGGAAAGCTTGTAATCTG-3' |
| exon 18 | 5'-TAGCTCTGTCAAACCTAACCT-3' | 5'-TAGTCTGACTTGAAACACTGC-3' |
| exon 19 | 5'-CCATAGACATTTGAGGTCACT-3' | 5'-ATGAAAACAAAGAGCGGACTT-3' |
| exon 20 | 5'-CAGATCCACAGCTTACATTAG-3' | 5'-AAAACATGAAGAGGGAGATG-3' |
| exon 21 | 5'-GTAAGAAATGTTATTTTTCAG-3' | 5'-CAATCCCACTGGTCCCTATTC-3' |
| exon 22 | 5'-GTAATTGGTAAAAGCGACTGT-3' | 5'-TTAAGTGTGCCTGTCTTGTGG-3' |
| exon 23 | 5'-CCACTGAAATGTCACGAAAGG-3' | 5'-TAACTGACAGAACCAGGCTAT-3' |
| exon 24 | 5'-ACCAACCACGCCACCCTGCTC-3' | 5'-TTTGTTCAACTCCCACTTATG-3' |
| exon 25 | 5'-AGGCTTCAGTAAGAGCATCTC-3' | 5'-AGCCCACTTTTAGGGGTTGGA-3' |
| exon 26 | 5'-CTGCTGGAATTCTAAAAACCT-3' | 5'-TCCCCATCCTTGTCTCTCATA-3' |
| exon 27 | 5'-GGAGGACTCACTCACTGTTCC-3' | 5'-TGCCATTTTATTAAGGACAAA-3' |
| exon 28 | 5'-TTGCATCAACTTTCCATCTTC-3' | 5'-CCCCTGTAACTGGTGCGTCAT-3' |

**Table 5. Polymorphisms detected in the six genes of interest with their allelic frequencies.**

| gene | position | polymorphism | allelic frequency (%) | | | | odds ratio (95% c.i.) |
|---|---|---|---|---|---|---|---|
| | | | patients (n = 58) | | healthy subjects (n = 61) | | |
| | | | r | v | r | v | |
| *ABCB*1 | exon 21 | 2677G > T/A | 45.5 | 54.5 | 50.8 | 49.2 | 1.24 (0.74-2.07) |
| | exon 26 | 3435C > T | 51.8 | 48.2 | 63.3 | 36.7 | 1.61 (0.95-2.72) |
| *ABCC*1 | exon 8 | 825T > C | 50.9 | 49.1 | 61.5 | 38.5 | 1.54 (0.92-2.59) |
| | exon 9 | 1062T > C | 50.9 | 49.1 | 61.5 | 38.5 | 1.54 (0.92-2.59) |
| | intron 9 | 1218 + 8 A > G | 50.9 | 49.1 | 61.5 | 38.5 | 1.54 (0.92-2.59) |
| | exon 13 | 1684T > C | 25.5 | 74.5 | 27.9 | 72.1 | 1.13 (0.63-2.03) |
| | exon 16 | 2007C > T | 98.2 | 1.8 | 93.4 | 6.6 | 0.26 (0.05-1.25) |
| | exon 17 | 2168G > A | 89.3 | 10.7 | 90.2 | 9.8 | 1.10 (0.47-2.56) |
| *ABCC*2 | promoter | -24C > T | 83.6 | 16.4 | 84.4 | 15.6 | 1.00 (0.49-2.01) |
| | exon 10 | 1249G > A | 82.8 | 17.2 | 85.2 | 14.8 | 1.20 (0.60-2.41) |
| | exon 18 | 2302C > T | 100.0 | 0.0 | 100.0 | 0.0 | - |
| | | 2366C > T | 99.2 | 0.8 | 100.0 | 0.0 | - |
| | exon 28 | 3972C > T | 81.0 | 19.0 | 82.0 | 18.0 | 1.06 (0.55-2.05) |
| | exon 31 | 4348G > A | 100.0 | 0.0 | 100.0 | 0.0 | - |
| *ABCC*3 | promoter | -1767G > A | 85.5 | 14.5 | 83.3 | 16.7 | 0.85 (0.42-1.74) |
| | exon 2 | 135G > T | 96.4 | 3.6 | 97.5 | 2.5 | 1.44 (0.32-6.60) |

 

Continued **Table 5**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *ABCG*2 | exon 4 | 376C > T | 97.3 | 2.7 | 98.4 | 1.6 | 1.65 (0.27-10.1) |
| | exon 5 | 421C > A | 71.4 | 28.6 | 73.8 | 26.2 | 1.13 (0.63-2.00) |
| *ABCB*11 | promoter | -15281_-15278 CTCT > delete | 62.9 | 37.1 | 74.6 | 25.4 | 1.73 (0.99-3.01) |
| | intron 3 | exon 3 + 84C > T | 92.2 | 7.8 | 95.1 | 4.9 | 1.63 (0.56-4.72) |
| | exon 4 | 108T > C | 73.3 | 26.7 | 71.3 | 28.7 | 0.91 (0.51-1.60) |
| | intron 4 | exon 4 + 188A > G | 100.0 | 0.0 | 99.2 | 0.8 | - |
| | intron 4 | exon 4 + 196T > C | 100.0 | 0.0 | 99.2 | 0.8 | - |
| | exon 5 | 270T > C | 92.2 | 7.8 | 95.1 | 4.9 | 1.63(0.56-4.72) |
| | exon 9 | 807T > C | 73.3 | 26.7 | 70.5 | 29.5 | 0.87 (0.50-1.54) |
| | exon 10 | 1030G > A | 100.0 | 0.0 | 99.2 | 0.8 | - |
| | intron 11 | exon 11 + 57C > A | 100.0 | 0.0 | 99.2 | 0.8 | - |
| | exon 13 | 1331C > T | 23.3 | 76.7 | 25.4 | 74.6 | 1.12 (0.62-2.03) |
| | intron 13 | exon 13 + 70T > C | 23.3 | 76.7 | 25.4 | 74.6 | 1.12 (0.62-2.03) |
| | intron 18 | exon 18 + 97A > G | 70.7 | 29.3 | 63.1 | 36.9 | 0.71 (0.41-1.22) |
| | intron 18 | exon 18 + 98T > C | 70.7 | 29.3 | 63.1 | 36.9 | 0.71 (0.41-1.22) |
| | intron 18 | exon 19 − 17C > A | 40.5 | 59.5 | 39.3 | 60.7 | 0.95 (0.57-1.60) |
| | exon 21 | 2594C > T | 99.1 | 0.9 | 99.2 | 0.8 | 1.05 (0.07-17.0) |
| | exon 24 | 3084A > G | 39.7 | 60.3 | 38.3 | 61.7 | 0.95 (0.56-1.60) |

In *ABCB*11 gene, numbering of nucleotides is according to the GenBank accession numbers AC008177.3 (promoter and exons 1–21) and AC069137.6 (exons 22–28). Positions of polymorphisms are counted with respect to the BSEP translation initiation site being +1 and the 5'-following base being -1. r: reference type, v: variant type.

**Table 6. Haplotype assessment and HCC.**

| *ABCC*1 haplotype pattern | frequency | |
|---|---|---|
| | patient | control |
| C C C C G | 0.37 | 0.23 |
| T T C C G | 0.25 | 0.36 |
| T T T C G | 0.19 | 0.19 |
| C C T C G | 0.07 | 0.07 |
| T T C C A | 0.06 | 0.00 |
| C C C C A | 0.05 | 0.08 |
| T T C T G | 0.02 | 0.05 |
| | $\chi^2 = 16.75$ ($p = 0.010$) | |
| *ABCB*11 haplotype pattern | frequency | |
| | patient | control |
| Del T T C G | 0.24 | 0.17 |
| C C C C G | 0.15 | 0.13 |
| C T T T G | 0.13 | 0.12 |
| C T T C A | 0.13 | 0.06 |
| C T T T A | 0.10 | 0.10 |
| C C C C A | 0.08 | 0.15 |
| Del T T C A | 0.06 | 0.05 |
| C T T C G | 0.06 | 0.19 |
| | $\chi^2 = 15.69$ ($p = 0.028$) | |

quently observed haplotype (0.24) in the patient group, while CTTCG was the major pattern (0.19) in the control group. The frequency of CTTCA was higher in patients than in controls (0.13 vs. 0.06), but the frequency of CCCCA was lower in patients than in control group (0.08 vs. 0.15).

We tested the association of all combinations of polymorphisms of different genes with HCC, and identified three as candidate markers for susceptibility to HCC (**Table 7**). All candidates were a combination of two of the following three efflux ABC transporters; *ABCB*1 (3435C > T), *ABCC*1 (825T > C) and *ABCB*11 (−15281_−15278 CTCT > delete). The frequencies of the three mutations in the three genes in both patients and healthy subjects obeyed the Hardy-Weinberg law, indicating normal distribution of the genetic mutations in the subjects of our study. The three combinations were: [*ABCB*1 and *ABCC*1, odds ratio (95% CI.) = 4.44 (1.13-17.5)], [*ABCB*1 and *ABCB*11, 4.47 (1.26-15.9)], and [*ABCC*1 and *ABCB*11, 3.79 (1.21-11.9)].

## 4. Discussion

Drug transporters can affect the absorption, distribution, and excretion of not only drugs but also mutagens and carcinogens, as well as their metabolites. The genetic polymorphisms in drug transporters have been linked to

 

**Table 7. Association of combination of efflux transporter polymorphisms with HCC.**

| combined polymorphism | patients (n = 58) | | healthy subjects (n = 61) | | odds ratio (95% c.i.) |
|---|---|---|---|---|---|
| | r | v | r | v | |
| *ABCB*1 3435C > T & *ABCC*1 825T > C | 3 | 30 | 12 | 27 | 4.44 (1.13~17.5) |
| *ABCB*1 3435C > T & *ABCB*11-15281_-15278CTCT > delete | 4 | 23 | 14 | 18 | 4.47 (1.26~15.9) |
| *ABCC*1 825T > C & *ABCB*11-15281_-15278CTCT > delete | 6 | 26 | 14 | 16 | 3.79 (1.21~11.9) |

We compared frequencies between subjects homozygotes for the reference allele (r) and subjects with at least one variant (v) allele in each gene.

inter-individual differences in PK/PD profiles of clinically relevant drugs [8], leading to expectations that these polymorphisms may also account for significant individual differences in susceptibility to exogenous and endogenous mutagenic and carcinogenic insults. In fact, in the clinical setting, some cases of HCV-related cirrhosis progress to HCC, while others do not. Among drug transporters, ABC transporters potentially affect the risk of carcinogenesis, because their substrates include carcinogens such as benzo[*a*]pyrene and p-glycoprotein, nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and MRP1 [23], and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and BCRP [24].

The pharmacogenomic association of 3435C > T in *ABCB*1 with renal epithelial tumors [25] and colorectal cancers has been studied [26]. However, to our knowledge, there are very few reports on its association with HCC. Although both previous studies indicated that the 3435T allele was a risk factor for developing cancers, we found no association between any SNP of any gene of interest and HCC in the present study, suggesting that it is difficult to predict HCC based on single SNP in a single gene (**Table 5**).

Next, we conducted a haplotype assessment and identified *ABCC*1 and *ABCB*11 as susceptibility genes of hepatic carcinogenesis. MRP1 transports various chemical compounds. Aflatoxin $B_1$ ($AFB_1$), a mycotoxin, is a contaminant of certain food products including grains and peanuts. $AFB_1$ has been shown to be carcinogenic in the lungs as well as the liver [27]. The high-affinity transport of glutathione conjugates of $AFB_1$ epoxide has been demonstrated using human MRP1-enriched membrane vesicles [28]. However, the incidence of tumors in *Mrp*1(-/-) versus *Mrp*1(+/+) mice 12 months after eight-week exposure to $AFB_1$ revealed that the expression of MRP1 provided no protection to the lungs or liver from the carcinogenicity of this compound [29]. Although $AFB_1$ might be a substrate for MRP1 *in vivo*, functionally redundant ABC transporters may compensate for MRP1 in MRP1-deficient animals. Since the level of MRP1 in human liver is relatively low in comparison with that in other organs (e.g., lungs and kidneys), [30] MRP1 is likely to act as a barrier in cooperation with other transporters in the liver.

Since chemotherapy has long been a standard treatment to reduce the growth of HCC, the development of multidrug resistance by ABC transporters is a major obstacle to its success. Bonin *et al*. [31] investigated the expression of MRP1, MRP2, MRP3, p-glycoprotein, and MDR3 in paraffin-embedded tissues by quantitative RT-PCR. They found that multidrug resistance proteins, in particular MRP1, MRP2 and p-glycoprotein, were increased in HCC. The extent of MRP1 expression was similar in neoplastic and perineoplastic tissue, but this was not the case for MRP2 and p-glycoprotein. The expression of MRP1 altered neoplastic proliferation with some correlation to malignancy [32].

Recently, Knisely *et al*. [33] studied the association between PFIC, diagnosed from immunohistochemical evidence of a deficiency of BSEP and a mutational analysis of the *ABCB*11 gene, and HCC in 11 unrelated children, and concluded that BSEP deficiency increases the risk of HCC in early life. Increased intracellular concentrations of bile acids have reported to be carcinogenic in human gastrointestinal cancer [34]. Although we had no PFIC patients and no young children, these reports support our findings of an association between the transport capability of BSEP and HCC.

In this study, after analyzing all the combinations of SNPs for all the transporter genes examined, three combinations of SNPs in two different genes were detected as candidate markers for susceptibility to HCC: 3435C > T in *ABCB*1 and 825T > C in *ABCC*1, 3435C > T in *ABCB*1 and -15281_-15278CTCT > delete in *ABCB*11, and 825T > C in *ABCC*1 and -15281_-15278CTCT > delete in *ABCB*11. Interestingly, all the genes encode

 

ABC transporters, suggesting that efflux transporting systems, which could be involved in protecting tissues from the accumulation of xenobiotics and resulting toxicity, play an important role in the development of HCC.

Among various SNPs in the *ABCB*1 gene, 3435C > T, a synonymous variant, has been investigated in greatest detail in humans [8,14]. Using an immunochemical approach to quantify P-glycoprotein content, Hoffmeyer *et al*. [35] found that 3435C > T was associated with a significantly reduced intestinal P-glycoprotein content in subjects with the T/T genotype. However, the association of 3435C > T with expression (both at the protein and mRNA levels) and *in vivo* transport activity (including human studies) is controversial, with increased, decreased, and unchanged expression/transport being reported [14]. Therefore, epigenetic concerns have been introduced into the experiments in order to assess the confounding results. Recently, Wang *et al.* [36] reported that the 3435C > T variant was associated with an allelic expression imbalance due to decreasing mRNA stability, leading to decreasing hepatic ABCB1 mRNA levels. On the other hand, Kimchi-Sarfaty *et al.* [37] reported that 3435C > T altered the substrate specificity of P-glycoprotein. They hypothesized that the variant changes the mRNA translation rate and thereby affected protein folding.

825T > C in *ABCC*1 and -15281_-15278CTCT > delete in *ABCB*11 were synonymous and promoter variants, respectively. Thus, none of the three candidate variants including 3435C > T in *ABCB*1 in the combination study had an influence on the amino acid sequence in each transporter protein. Similar to 825T > C, the effect of -15281_-15278CTCT > delete on transport activity has not yet been well investigated. One luciferase gene reporter experiment indicated that promoter constructs including -15281_-15278CTCT > delete were not associated with changes in luciferase activity [18]. All three candidate variants could be detected at relatively high frequency even in the healthy subjects, casting doubt on a significant contribution to the risk of developing HCC; however, a role for these variants in HCC cannot be excluded. Based on the present findings, a single mutation may not be involved in HCC under normal conditions, but combined with another mutation, may increase susceptibility to HCC. Indeed, there is substantial evidence that p-glycoprotein and MRP1 have overlapping functions in tissue defense [5]. Although each transporter alone would not have sufficient transport capability, together they are likely to provide protection against the accumulation of carcinogen(s).

Some carcinogenic substances may undergo activation or inactivation by hepatic enzymes. This may introduce a bias in the study, because the difference attributed to ABC-transporters may arise in fact from metabolizing enzymes. Further studies including polymorphisms of genes encoding hepatic metabolizing enzymes such as cytochrome P450 (CYP) 2D6 and CYP3A5 are required.

There are some limitations of the present study. We genotyped various SNPs that have been shown to be most functionally significant in previous pharmacogenomic studies or observed at relatively high frequencies without ethnic diversity. However, since the genotypes investigated here were not based on known phenotypes, the usefulness of the results may be limited at this stage. Furthermore, the numbers of enrolled patients and healthy subjects were small (around sixty), and the study had insufficient statistical power to detect even though a large differences in gene frequency between the two groups. Therefore, this study represents a preliminary comparison of frequencies of possible SNPs. Further studies to confirm the association of the SNPs with HCC and how these polymorphisms are associated with phenotypic differences are warranted in a large population study."

In conclusion, the present study suggests that some SNPs of the ABC transporter genes such as *ABCB*1, *ABCB*11, and *ABCC*1 are associated with the susceptibility of developing HCC, implying that aberrant hepatic clearance of toxic substances may increase the risk of hepatocarcinogenesis.

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# Synthesis, Spectroscopic, and *in Vitro* Cytotoxic Studies of Fatty Acid Analogues of 2, 6-Diisopropylphenol

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## ABSTRACT

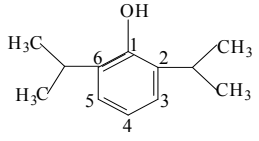
*This paper represents the synthesis, spectral analysis and in-vitro cytotoxic studies of some novel fatty acid anti-cancer conjugates of* 2, 6-*diisopropylphenol* (*propofol*). *Propofol is a potent intravenous hypnotic agent which is widely used for the induction and maintenance of anesthesia and for sedation in the intensive care unit. Propofol also possess anti-cancer properties in addition to its sedative effects. Cytotoxicity of all the synthesized compounds was examined against human HeLa cancer cell lines. The anti-cancer screening of these novel drug candidates suggest that all of them reported here may be useful for the treatment of cancer as all of them exhibited significant anticancer activity against human HeLa cancer cell lines. The results indicate that these novel drug candidates might represent a new class of anticancer agents.*

***Keywords*:** *Propofol, Anesthetic, Sedative,* 10-*Undecenoic Acid, Cytotoxicity*

## 1. Introduction

Cancer is the leading cause of death among the global population including United States. A recent American Cancer Society statistical survey has concluded that cancer now exceeds heart disease as the top cause of death among Americans below the age of 85 years, responsible for about 47600 fatalities compared with 450,600 deaths yearly from heart disease. Cancer refers to a large number of diseases categorized by unregulated replication (proliferation) and spread (metastasis) of abnormal cells. Surgery, chemotherapy and radiation therapy are the only methods for treating cancer patients. Infact, chemotherapy is the most popular therapeutic method. Therefore, there is a need to develop new anticancer drugs having better efficiency and broad spectrum activity.

2,6-diisopropylphenol or propofol (**Figure 1**) a short-acting intravenous anesthetic agent [1,2], has gained wide acceptance since its introduction in the late 80s, not only in operating rooms but also in other departments, due to its several advantages. Apart from its multiple anesthetic advantages, it has been reported recently that propofol exerts a number of non-anesthetic effects. It is a potent antioxidant [3-5] and has been shown to stimulate protein kinase C [6,7]. It inhibits calcium entry in muscle cells [8] and increase the calcium sensitivity of myofilaments in ventricular myocytes [9]. It decreases cerebral oxygen consumption, reduces intracranial pressure and has potent anti-convulsant properties [10]. It is a potent bronchodilator and has anti-inflammatory properties [11]. It has immunomodulatory analgesic, antiemetic and neuroprotective effects. It also exerts direct inhibitory effects on recombinant cardiac sarcolemmal KATP channels [12]. The antioxidant activity of propofol together with the stimulating effect of protein kinase C suggests that propofol might have the potential to modulate apoptosis. 3 to 8 μg/ml concentrations of propofol were reported to decrease the metastatic potential of human cancer cells, including HeLa, H71080, HOS and RPMI-7951 cells [13].




2,6-diisopropylphenol

**Figure 1. Structure of 2, 6-diisopropylphenol (propofol).**

Propofol was reported to inhibit pulmonary metastasis of murine osteosarcoma (CM8) cells in mice through the modulation of Rho A [14]. R. Siddiqui *et al*. [15], studied the effect of two omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), combined with propofol on a breast cancer cell line in vitro. The results of the study showed that propofol and DHA or EPA has a much more significant effect on cancer cells when used in combination, as conjugates, than when used alone. The conjugates inhibit cancer cell adhesion by 15% and 30% respectively, reduce cell migration by 50% and increase apoptosis by 40%. Harvey *et al*. [16] reported the characterization and evaluation of novel anti-cancer conjugate, 2, 6-diisopropylphenol-docosa-hexaenoate (PP-DHA) and its analogs on breast cancer cell lines. Results suggested that these novel conjugates and their amide derivatives may be useful for the treatment of breast cancer.

Current trends in the treatment of human cancers favor drug combinations that result in improved responses, where the contributions of a variety of fatty acids have been proved highly significant [17,18] A number of fatty acids are part of our diet; therefore, nutritional dietary supplements highly enriched in certain fatty acid have been suggested to prevent the side effects of cancer therapy [18,19]. Certain triglycerides and fatty acids have the potential to prevent or inhibit carcinogenesis [20-23].

10-undecenoic acid is an eleven-carbon monounsaturated fatty acid, a substance found naturally in the body (occurring in sweat); undecenoic acid is produced commercially by the vacuum distillation of castor bean oil, via the pyrolysis of ricinoleic acid. It is used in the manufacture of pharmaceuticals, cosmetics and perfumery including anti-dandruff shampoos, anti-microbial powders and as musk in perfumes and aromas. It is an inexpensive source for the synthesis of pheromones of cotton pests, peach tree borer and cherry tree borer [24]. It is an economical antifungal agent and is the active ingredient in many topical over-the-counter antifungal preparations. [25] 10-Undecenoic acid has long been known to be fungicidal against *Candida albicans*, thus helping achieve a healthy balance of normal vaginal and intestinal flora [26]. It is FDA approved in over the counter medications for skin disorders or problems. It is the active ingredient in medications for skin infections, and relieves itching, burning and irritation. It is also used in the treatment of psoriasis [27]. 10-Undecenoic acid is also proven to have antibacterial and anti-viral properties that are effective on viral skin infections such as the Herpes simplex virus [28,29]. It has the bifunctionality, odd carbon number and the position of its unsaturated double bond at the end of the chain, makes it a versatile tool for chemical synthesis. Although most studies of 10-Undecenoic acid have concentrated on its anti-fungal, anti-bacterial, anti-viral activities, there are a few reports indicating that the derivatives of this compound may also affect cellular processes related to cancer [30,31]. Mustafa *et al*. [32] reported first synthesis and antitumor evaluation of F, N, and S containing $C_4$ α-fatty acid derivatives of podophyllotoxin. The efficient synthesis of a series of Fatty acid-based derivatives of propofol possessing Sulfur atom (**Figure 2**) and their significant *in vitro* selectivity for inhibiting the growth of cancer cells (HeLa cancer cell lines) have been demonstrated here. The results indicate that these novel conjugates might represent a new class of anticancer agents.

## 2. Materials and Methods

A thin layer chromatographic applicator (Toshniwal, India), 20 × 3.5 cm glass plates and 24 × 6 cm glass jar were used for performing thin layer chromatography (TLC). Silica Gel "G" (E. Merck, India) was used as a stationary phase. Petroleum ether and diethyl ether (1:1, vol/vol) was used as a developing solvent. Reaction products on TLC plates were visualized by UV light and by exposure to iodine vapors. Column chromatographic separations were performed using silica gel "G" packing of particle size 60-120 mesh (petroleum ether/diethyl ether, 1:1, v/v). $H^1$NMR and $C^{13}$NMR spectra were recorded on Advance DRX-200 Bruker, (Switzerland) NMR Spectrometer. Molecular weights were determined by MS route JMS-600H, Jeol (Japan) Mass Spectrometer. FTIR Spectra were recorded in chloroform on a Spectrum RX-1 FTIR, Perkin Elmer Spectrometer. All these analyses were done in CDRI (Central Drug Research Institute, Lucknow), India. 2, 6-diisopropyl phenol (DPP), 4-dimethyl amino pyridine (DMAP) was procured from Acros chemicals. The coupling reagent-N, N-dicyclohexyl carbodiimide (DCC) was purchased from Fluka chemical corporation (New York), 10-undecenoic acid and β-mercaptoethanol was purchased from Aldrich Chemicals and methylene chloride was purchased from CDH Chemicals (Mumbai, India).

### 2.1. Synthesis of Compound, 1-Isopropyl (11'-Mercaptoethanol) Undecanoate (S-1)

β-Mercaptoethanol (1 mmol) and 10-undecenoic acid (1 mmol) were dissolved in dry dichloromethane (5 ml) and stirred at room temperature. Progress of reaction was monitored on TLC plates. This reaction showed the formation of single product and was completed in 10 hours. Methylene chloride was rotary- evaporated and the residue was passed through a silica gel column 60-120 mesh particle size, (petroleum ether/diethyl ether, 1:1, v/v; $R_F$ = 0.2) to obtain a solid white powder (yield 90%). To a stirred solution of the above product (1mmol) in methy-

lene chloride (5 ml) was added 2, 6-diisopropyl phenol (1 mmol) and DMAP (catalytic amount). The reaction mixture was allowed to stir at room temperature under nitrogen for 10 minutes before DCC (1 mmol) was added

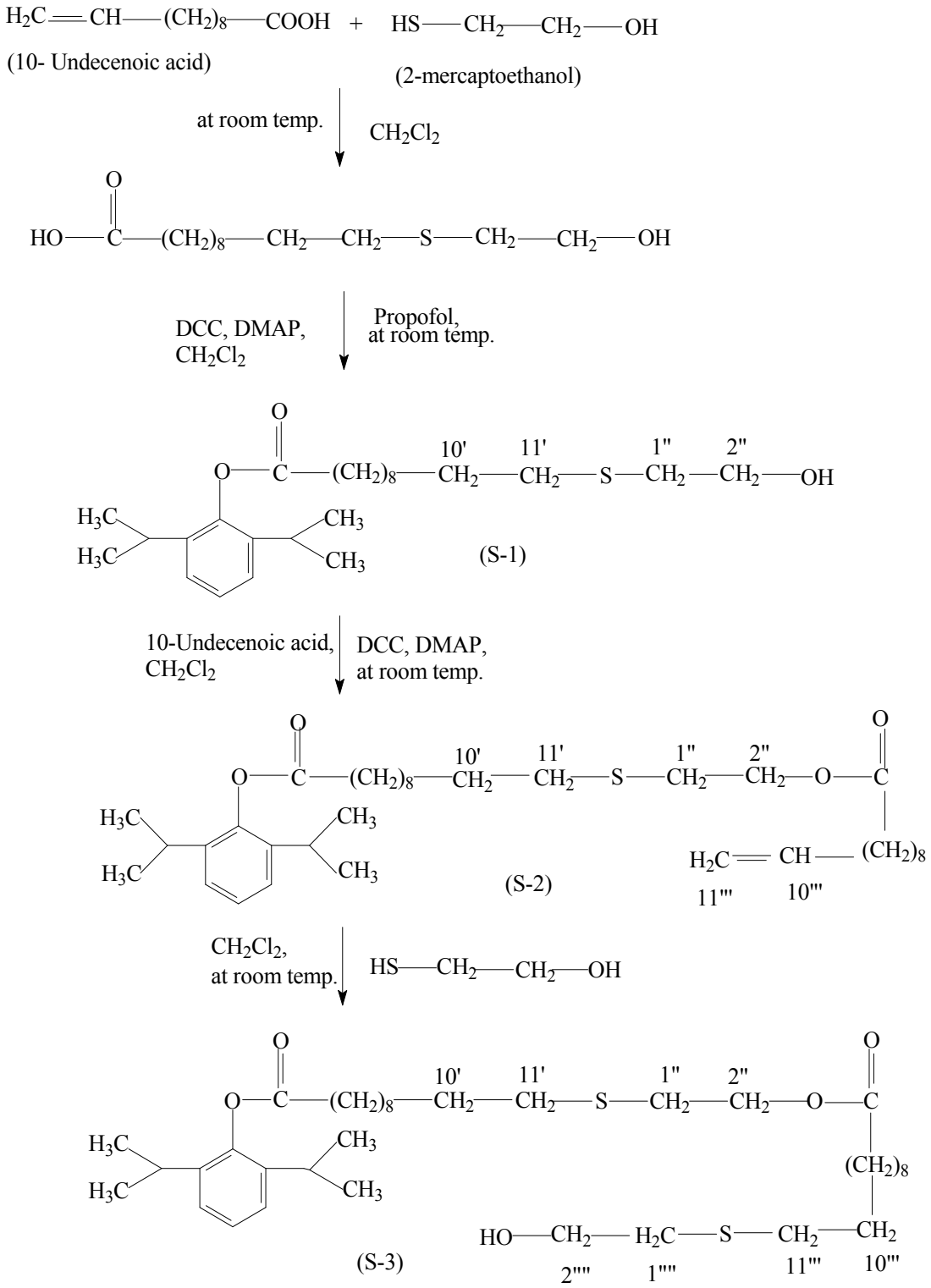



**Figure 2. Reaction scheme.**

to it. The reaction mixture was allowed to stir at room temperature. Progress of reaction was monitored on TLC plates. This reaction also showed the formation of single product and was completed in 7 hours. The reaction mixture was filtered to remove solid dicyclohexylurea and the filtrate was evaporated under reduced pressure at 20°C. The semisolid mass was purified by column chromatography on silica gel, 60-120 mesh particle size, (petroleum ether/diethyl ether, 1:1, vol/vol; $R_F$ = 0.8) to obtain S-1 (950 mg, 90%) as a sticky, viscous, colorless oil.

### 2.2. Synthesis of Compound, 1-Isopropyl (11'-Mercaptoethane-Undec-10"'-Enoate)-Undecanoate (S-2)

Equimolar amounts of the above compound and 10-Undecenoic acid and catalytic amounts of DMAP were dissolved in 5 ml of methylene chloride and stirred at room temperature for 10 minutes before DCC was added to it. The reaction mixture was allowed to stir at room temperature. Progress of reaction was monitored on TLC plates. This reaction also showed the formation of single product and was completed in 12 hours. The reaction mixture was filtered to remove solid dicyclohexylurea, and the filtrate was evaporated and the residue was purified by column chromatography on silica gel, 60-120 mesh particle size (petroleum ether/diethyl ether, 1:1, vol /vol; $R_F$ = 1.0) to obtain S-2 (1.9 g, 95%) as a sticky, viscous, colorless oil.

### 2.3. Synthesis of Compound, 1-Isopropyl (11""-Mercaptoethanol)-11'-Mercaptoethane -Diundecanoate (S-3)

Equimolar amounts of the above compound and β-mercaptoethanol were dissolved in 5 ml of dry methylene chloride. The reaction mixture was allowed to stir at room temperature and the progress of reaction was monitored on TLC plates. This reaction also showed the formation of single product and was completed in 6 hours. Methylene chloride was rotary evaporated and the residue was purified by column chromatography on silica, 60-120 mesh particle size (petroleum ether/diethyl ether, 1:1, vol/vol; RF = 0.7) to obtain S-3 (1.0 g, 90%) as a sticky, viscous and colorless oil.

### 2.4. Assay for Anti-Cancer Activity

The compounds S-1, S-2, S-3 were examined for their *in vitro* cytotoxicity against human cancer cell line HeLa cell lines. The cells were cultured in serum free RPMI-1640 medium supplemented with antibiotic solution (60 mg/L), at 37°C, 95% humidity, 5% $CO_2$ and sub cultured every 2–3 day using standard cell culture techniques. For the assay, cells from different cell lines were seeded in 6-well plates at a density of $1 \times 10^6$ cells/well and were allowed to grow undisturbed for 24 h before addition of the test compound. Different concentrations of compound were added and cells were further incubated for 24 h. After the incubation time the samples were diluted in RPMI-1640 medium at 37°C. Cytotoxicity was made by using the trypan blue assay [33] to determine cell viability. The results are summarized in **Figure 3**.

## 3. Results and Discussion

Here, the efficient synthesis of C1α-Fatty acid (FA) analogs of Propofol containing the Sulfur atom has been reported. The terminal double bond (C-11/10) in the side chain of 10-undecenoic acid underwent an efficient addition reaction at C-11 when treated with a slight excess of 2-mercaptoethanol to give a saturated thioether-incor-

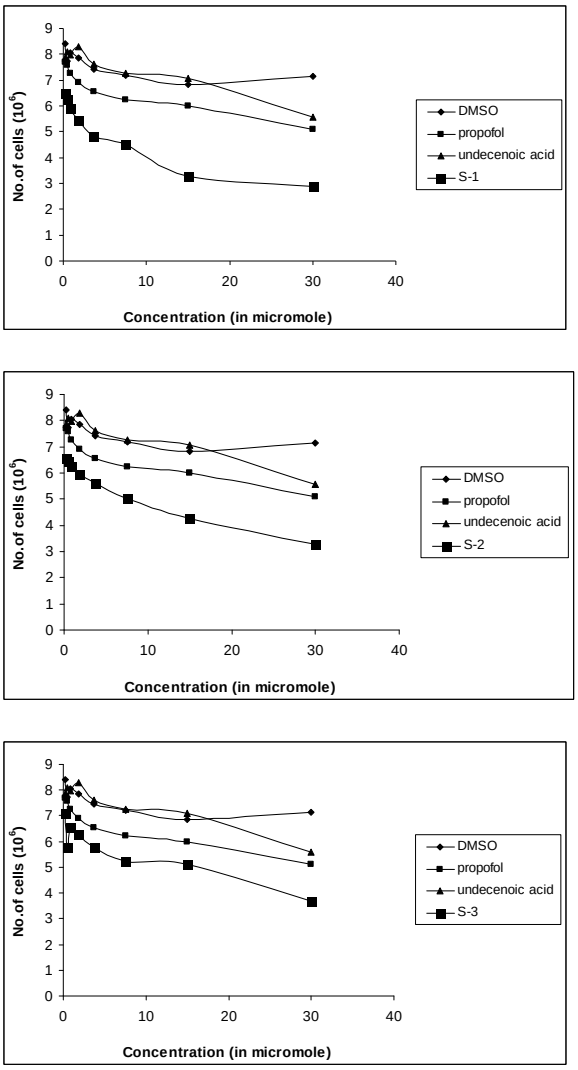



**Figure 3. Graphs showing variation of number of viable cells with concentration (in micromole) of S-1, S-2 and S-3.**

porated analog S-1, after reacting with the hydroxyl group of propofol. DCC (N, N-dicyclo hexyl carbodiimide) and DMAP (4-dimethyl amino pyridine) was used to esterify the 1α-hydroxy group of propofol with the carboxylic acid group of fatty acid, 10-Undecenoic acid. Similarly, S-2 compound was prepared by the esterification of hydroxyl group of S-1 compound with carboxylic acid group of fatty acid, undec-10-enoic acid in the presence of DCC and DMAP. Then, S-3 compound was synthesized by the addition reaction at the terminal double bond (C-11'''/10''') in the side chain of compound S-2 when treated with the slight excess of 2-mercaptoethanol. The reaction scheme is shown as **Figure 2**. The stereochemistry of all the stereogenic centers (C-1 of propofol, C-11' of fatty acid unit, C-2" of 2-mercapto- ethanol and C-11"' of second unit of fatty acid) at which the coupling reactions took place, was retained. Spectroscopic data established their chemical structures and are summarized in experimental section.

The IR spectra of compound S-1 show a strong and broad absorption bands at 1738.2 and 1165.3 $cm^{-1}$ which are attributable to C=O and C-O bands respectively, and indicate the presence of an ester. The carbon signals at δC 156.76 and 173.63 confirmed the presence of an ester group. A strong band at 3034.8 $cm^{-1}$ is characteristic of an aromatic C-H (propofol) and the band at 2930.4 and 2856.7 $cm^{-1}$ is characteristic of aliphatic C-H bonds. A strong band at 3361.8 $cm^{-1}$ confirm the presence of the hydroxyl group (OH) which is further correlated with a carbon signal at δC 70.1. The chemical shifts for the three aromatic protons are moved downfield at ∂H 6.897 (t, J = 7.6 Hz, 1H), 7.06 (d, J = 7.6 Hz, 1H) and 7.14 (d, J = 6.4 Hz, 1H) and their respective carbon signals appeared at δC 120.56, 123.4 and 123.83. For twelve protons of the two isopropyl groups of propofol, two doublets were observed at ∂H 0.892 (d, J = 6.8 Hz, 6H) and 1.20 (d, J = 6.8 Hz, 6H) and their respective carbon signals appeared at δC 24.70 and 24.92. Two multiplets were observed at ∂H 2.92 (m, 1H) and 3.18 (m, 1H) for the protons of the carbon atoms adjacent to the C-2 and C-6 of propofol. The carbon signals at δC 27.51 and 63.35 correspond to 1"CH2 and 2"CH2 and their respective chemical shifts at ∂H 2.68 (t, J = 6.0 Hz, 2H), 3.68 (t, J = 6.0 Hz, 2H). Signals from 1.26-1.32 (m, 12H) correspond to the 6 X CH2' groups of fatty acid chain, 12H. The chemical shift at ∂H 2.04 (m, 4H) attribute to 3'CH2 and 9'CH2. Similarly at ∂H 2.34 (m, 2H) relate to 2'CH2 of fatty acid chain and chemical shift at ∂H 2.58 (t, J = 7.6 Hz, 2H) correspond to 11'CH2 of fatty acid chain.

The IR spectra of compound S-2 show a broad and strong band at 1718.2 $cm^{-1}$ and 1216 $cm^{-1}$ correspond to the C=O and C-O bands respectively, and indicate the presence of an ester group which is correlated to the carbon signals at δC 172.3 and 173.56 that confirmed the presence of two carbonyl groups (ester and estolide carbonyls). Estolides are a group of FA polyesters resulting from ester bond formation between the oxygen of a hydroxyl group of one FA and the carbonyl carbon of the terminal carboxylic group of another FA. A distinct absorption band at 1642.3 $cm^{-1}$ represent alkenes which is further confirmed by the chemical shifts of the olefin protons (10'''H and 11'''H) at ∂H 4.95 (m, 2H), 5.78 (m,1H) and their respective carbon signals at δC 126.34 and 133.79. A strong band at 3020.1 $cm^{-1}$ is characteristic of an aromatic C-H (propofol) and the band at 2930.4 and 2855.2 $cm^{-1}$ is characteristic of aliphatic C-H bonds. Absence of absorption band at 2555 $cm^{-1}$ (-SH) attributed to the cleavage of –SH bond and the formation of new bond. The chemical shifts for aromatic protons are moved downfield at ∂H 6.90 (t, J = 7.5 Hz, 1H), 7.07 (d, J = 7.5 Hz, 1H), 7.16 (d, J = 6.9 Hz, 1H) with their respective carbon signals at δC 120.53, 123.36 and 123.81. For twelve protons of the two isopropyl groups two doublets were observed at ∂H 1.18 (d, J = 6.9 Hz, 12H) and their carbon signals appear at δC 23.68 and 24.68 respectively. Two multiplets were observed at ∂H 2.9 (m, 1H), 3.20 (m, 1H) for the protons of the carbon atoms that are adjacent to the C-2 and C-6 of propofol which is further confirmed by their respective carbon signals at δC 25.52 and 25.1. Two significant signals at δC 27.49 and 63.35 correspond to 1"CH2 and 2"CH2 and their respective chemical shifts appear at ∂H 2.64 (t, J = 6.0 Hz, 2H) and 3.87 (t, J = 6.0 Hz, 2H). Signals from ∂H 1.25-1.38 (m, 20H) correspond to the 6 X CH2" and 4 X CH2''' of fatty acid methylene chain, 20H.

The IR spectra of compound S-3 show a broad and strong absorption band at 1736.6 $cm^{-1}$ and 1165.3 $cm^{-1}$ which are attributable to C=O and C-O bands respectively, and indicate the presence of an ester. The carbon signals at δC 167.76 and 170.1 characterized the presence of two carbonyl groups (ester and estolide carbonyls). A strong band at 3073.9 $cm^{-1}$ is characteristic of an aromatic C-H (propofol) and the bands at 2930.2 and 2857.1 $cm^{-1}$ is characteristic of aliphatic C-H bonds. A strong band at 3409.8 $cm^{-1}$ confirmed the presence of a hydroxyl group and its respective carbon signal appeared at δC 69.12. The chemical shifts for the three aromatic protons are moved downfield at ∂H 6.576 (d, J = 5.4 Hz, 1H), 6.8 (t, J = 7.5 Hz, 1H), 7.06 (d, J = 7.5 Hz, 1H) and their respective carbon signals at δC 120.56, 123.4 and 123.86. For twelve protons of the two isopropyl groups two doublets were observed at ∂H 1.18 (d, J = 6.9 Hz, 1H) and 1.25 (d, J = 6.9 Hz, 1H) and their respective carbon signals appeared at δC 24.67 and 24.87. Two multiplets were observed at ∂H 2.9 (m, 1H) and 3.179 (m, 1H) for the protons of the carbon atoms that are adjacent to the

C-2 and C-6 of propofol which is further confirmed by their respective carbon signals at δC 26.33 and 25.52. Two significant signals at δC 27.49 and 63.4 correspond to 1"CH2, 1""CH2 and 2"CH2, 2""CH2 and their respective chemical shifts appear at ∂H 2.73 (double t, J1 = 6 Hz, J2 = 6.2 Hz, 4H) and 3.73 (t, J = 6 Hz, 2H), 3.89 (m, 2H). Signals from 1.25 to 1.41(m, 24H) correspond to the 6 X CH2'' and 6 X CH2''' groups of fatty acid methylene chain, 24H.

### 3.1. *In-Vitro* Anti-Cancer Activity

The compounds S-1, S-2, S-3 were examined for its *in vitro* cytotoxicity against human cancer cell line HeLa cell lines. A number of studies have concluded that chemically modified FA molecules possess more specific and potent biological activity with possible changes in their therapeutic targets [34]. The present work is based on the chemically transformed FA analogs of propofol. The growth inhibition graphs of S-1, S-2 and S-3 are shown in **Figure 3**.

All of these analogs showed promising cytotoxicity against human HeLa cancer cells tested. The FA-S-ether analog, *i.e.*, S-1, was functionalized with a fatty acid moiety (C-11) at C1α of propofol, which a close resemblance to the ω-hydroxy fatty acids has showed promising anti-cancer activity against human HeLa cancer cell lines. Mustafa *et al*. [35] reported that ω-hydroxy fatty acid analogs are excellent growth inhibitors of tumor and their anticancer activity increases with a decrease in the chain length of fatty acid. Same is the case here, S-1 and S-3 both possessed hydroxyl group just like ω-hydroxy fatty acids, but S-1 is more cytotoxically active than S-3. This is because of the reason that compound S-1 has got shorter fatty acid chain length as compared to compound S-3 which has longer chain length of fatty acid and hence comparatively possessed lesser anticancer activity than S-1. Compound S-2 showed more significant anti-cancer activity as compared to S-3 as it possessed terminal double bond in its fatty acid side chain. It showed significantly high growth inhibition activity in the HeLa cancer cell line. Both S-1 and S-2 were excellent growth inhibitors of HeLa cancer cell lines and S-3 is the least active analog of propofol.

The present investigations have indicated that the presence of hydroxyl group and unsaturation may be responsible for such activity. The above results demonstrated that all of these propofol FA conjugates (S-1, S-2, and S-3) not only inhibited cellular proliferation of HeLa cells but also decreased their viability which was confirmed by cell viability assay using trypan blue dye exclusion method. These results suggest that the propofol-fatty acid conjugates possessing Sulfur atom are far more effective at inducing apoptosis in HeLa cancer cells than are the unconjugated parent compounds, *i.e.*, 2, 6-diisopropyl phenol and 10-undecenoic acid.

## 4. Conclusions

The spectral analysis and anti-cancer screening of these novel compounds suggest that all of them reported here may be useful for the treatment of cancer as all of them show significant anticancer activity against human HeLa cancer cell lines. The conjugates are far more active than the parent compounds and possess unique anticancer activity. These novel drug candidates might represent a new class of anti-cancer agents. Further investigations of these novel fatty acid conjugates of propofol may provide useful leads in the development of new and effective pharmaceutical products. Experiments are under way to test these conjugates on different cancer cells lines and also in model systems *in vivo*.

## 5. Acknowledgements

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# Chest Wall Reconstruction with Precontoured Locking Plate Proof of Concept

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## ABSTRACT

*Complications after chest wall resection are common and are reported to occur in approximately 40% of patients. The most frequent complications are respiratory or wound complications. Restoring rib continuity after a resection is likely to prevent respiratory complications. However many patients remain painful after a reconstruction. This article describes a new technique using a titanium alloy precontoured locking plate and locking screws to reconstruct the chest wall after resection of the 7th rib on the left.*

***Keywords*: *Chest Wall Resection and Reconstruction***

## 1. Introduction

Complications after chest wall resection are common and are reported to occur in 37% to 46% of patients. Respiratory complications are most frequent, ranging from 20% to 24% [1] Wound infections occur in 5% [1] to 11% [2]. Furthermore many patients experience chronic pain. Restoring rib continuity after a resection is likely to prevent respiratory complications. Possible ways to reconstruct after chest wall resection are approximating adjacent ribs for small resections, or using non-rigid reconstructions like a polypropylene or vicryl mesh, or rigid reconstructions like polypropylene mesh/methylmethacrylate composite. This article describes a new technique by using a titanium alloy precontoured locking plate and locking screws to reconstruct the chest wall.

## 2. Case Report

Three years after a nephrectomy on the right side, because of a clearcell renal carcinoma, a 73-year-old female was diagnosed with a solitary metastasis in the left 7th rib. This metastasis was discovered on a CT-scan (**Figure 1)**, that was made because of recurring urinary tract infections and pain on her left thorax side. The patient gave informed consent for publication of this article.

The skin and fat was incised over the 7th rib on the left, with the patient lying on her right sight. The latissimus dorsi and serratus anterior muscles were partially opened and the tumor was exposed. The diameter of the tumor was approximately 6 cm and invaded the pleura. The rib was cut in healthy bone tissue on either side of the tumor and the tumor was excised including pleura. The continuity of the rib was restored by using a titanium precontoured locking plate and locking screws (**Figure 2**). The 6th and 8th rib were approximated using a double Vicryl. A thoraxdrain was left because of the pleural defect. The muscles were approximated using PDS, then subcutis and skin were closed.

The patient was extubated immediately after the operation. Painmedication consisted of a epidural catheter for 3 days. The thoraxdrain was removed 3 days after the operation and she left the hospital 4 days later in good condition.

At 2-months follow-up she had no pain, good thorax excursions and had picked up her daily activities.

## 3. Conclusions

Titanium is a strong, light and nonferromagnetic material with low density, causing little interference on computed tomography. The precountered plate and locking screws used in this case provided a stable reconstruction. The patient had no respiratory or wound complications, made a quick and full recovery, and reported no pain. It is therefore an effective way of restoring rib continuity after a resection. It should be considered as a proof of concept. Further investigation is required to proof it is also effective for multiple rib resections.

 

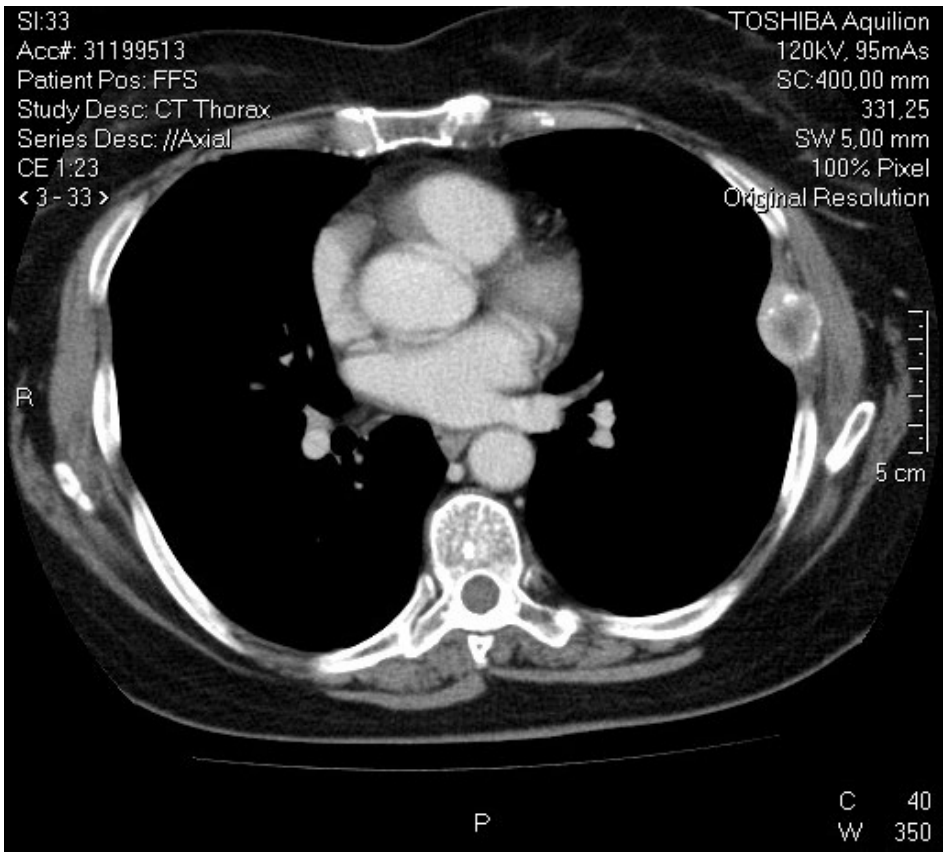


**Figure 1. CT-scan showing metastasis in the 7th rib left.**

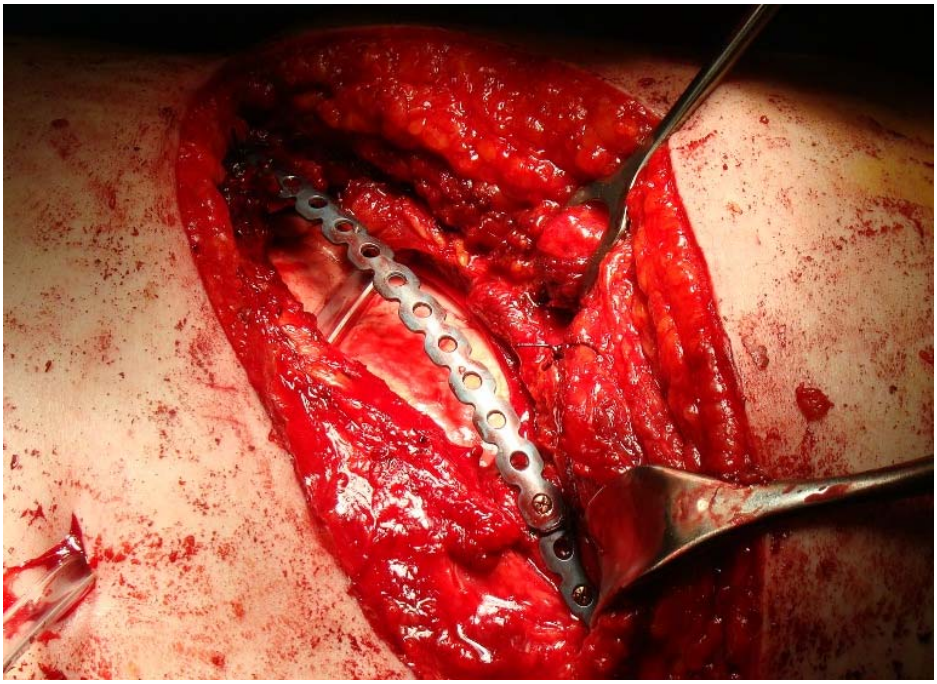

**Figure 2. rib reconstruction using a titanium precontoured locking plate and locking screws.**

 

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# HLA-Identical Dendritic-Leukemic Cell Hybrids Generate Specific CTLs *in Vitro*

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## ABSTRACT

*While being instrumental in the treatment of leukemic relapse after allogeneic hematopoietic stem cell transplantation, the impact of donor lymphocyte infusion* (*DLI*) *and its effectiveness remain debatable. Consequently it is widely accepted that more efforts are needed in order to make DLI more effective. This communication thus deals with the generation of specific CTLs in the clinical setting of HLA matched hematopoietic stem cell transplantation, to be used as an improved DLI treatment for post-transplantation relapsed leukemias. We assessed the potential of fused dendritic cells from donor origin, with leukemic cells from the HLA matched recipient for the generation of donor anti-tumor CTLs. Leukemic cells and donor dendritic cells were fused using polyethylene glycol* (*PEG*). *The hybrids were analyzed for double phenotype of both DC and tumor, and used for the education and generation of cytotoxic donor lymphocytes. Results demonstrate that efficient and specific CTLs can be generated and used in vitro for the elimination of the recipient tumor cells. These results form the basis for the establishment of a novel methodology aimed at generating active or passive anti-leukemic vaccine in relapsed patients.*

***Keywords*: *Dendritic Cells, Cytotoxicity, Cellular Therapy***

## 1. Introduction

Cancer immunotherapy is hampered by the fact that tumor specific antigens are either unknown, non-immunogenic or that the tumor develops means to evade the host immune response [1-3]. In attempting to circumvent these obstacles, novel approaches of cancer immunotherapy based on the development of potent tumor vaccines are extensively explored [4]. The use of dendritic cells (DCs), in that respect, has recently gained a remarkable momentum, both in pre-clinical and clinical settings [5-8].

Dendritic cells are potent antigen presenting cells (APCs) due to the expression of MHC I/II, co-stimulatory and adhesion molecules that provide secondary signals for the generation of primed T cells [9-11]. The introduction of tumor specific antigens into dendritic cells leading to the generation of cytotoxic T Lymphocytes (CTLs) against malignant cells has already been proven to be effective [4,12]. Thus, DCs can be pulsed with liposomal DNA, tumor cells or apoptotic cell fragments, peptides eluted from tumor cells, membranes, lysates, RNA and more [13-18]. Such strategies have generated positive in vitro immune responses directed against the relevant tumor cells. Nevertheless a more promising approach to the induction of primary anti-tumor immunity is through the generation of fusion between tumor cells and DCs [13,6] that use primarily whole tumor cells as the source of pulsing antigen. Indeed, cancer immunotherapy by DC/tumor cell fusion hybrids using solid carcinoma cells, in mouse models or in vitro with human cells such as breast and ovarian carcinoma, has been shown to elicit potent anti tumor effects via the induction of immune responses against multiple tumor-associated antigens [14,19-21].

The therapeutic potential of tumor-DC fused cells has been tested in phase I/II clinical trials in patients with melanoma and breast cancer [19,22]. The results demonstrated the safety of the vaccine although the therapeutic efficacy of such a procedure was shown to be variable but promising.

Immunotherapy of hematological malignancies such as myeloid leukemias is of particular complexity since very

few tumor antigens are known in those tumors [6,23]. Presently, the most clinically meaningful immunotherapeutic modality in hematological malignancies remains allogeneic stem cell transplantation followed, upon relapse, with the administration of allogeneic, donor derived T lymphocytes (DLI), aiming to generate a clinically significant graft-versus-leukemia (GVL) response [24-31]. Although effective in the chronic phase of chronic myelogenous leukemia (CML), DLIs have been of limited effectiveness in patients with other hematological malignancies, such as ALL principally due to the high proliferative rate of the tumor cells and their relative resistance to immunotherapy. Hence, additional approaches to improve DLI effectiveness are necessary. These may include the use of immunomodulatory cytokines, ex-vivo priming of the donor lymphocytes to the relevant tumor antigens and infusion of alloreactive NK cells (reviewed by Soiffer 2008) [32].

The use of DC-tumor hybrids has also been assessed in hematological malignancies, holding promise as a cellular vaccine for some of them [33,7,34]. For example, the fusion of AML myeloblasts and autologous DCs [7] or myeloma cells and allogeneic dendritic cells [34], were demonstrated to generate in vitro potent cytotoxic lymphocytes.

Nevertheless, the DC-tumor hybrid approach in HLA-matched allogeneic stem cell transplantation has never been assessed, particularly in the context of post relapse DLI treatment. It is expected that such a procedure may lead to the induction of very potent anti tumor CTLs selected in vitro for no-GVH activity. In addition, these fused cells could also serve as active and potent cell vaccine for pre-emptive immunization of patients at high risk for relapse. These issues represent the subject matter of our research and communication.

## 2. Materials & Methods

The study was approved by the Institutional Helsinki committee, and appropriate informed consents were obtained from all patients and healthy donors.

*Collection of cell samples*

Leukemia cell samples from blood or bone marrow were obtained from newly diagnosed patients with acute leukemia treated in our department. Peripheral blood lymphocytes were collected form their prospective matched HLA donors by direct venipucture or collected by lympho-pheresis (COBE Spectra, COB BCT, Lakewood, CO USA).

Mononuclear fractions were isolated using Ficoll-Hypaque (FH) separation, washed and cryopreserved in liquid nitrogen (using 10% DMSO as cryo-protectant), according to the standard method. If needed, a positive selection of leukemic cells with the relevant membrane marker beads (such as CD34) was performed in order to obtain a pure population of leukemic cells.

*Preparation of immature DCs in vitro*

Immature donor dendritic cells were derived from the mononuclear fraction by magnetic beads purification of CD14 positive cells using the Miltenyi MS-MACS isolation column (Miltenyi Biotec, Bergisch Gladbach, Germany). This enriched population was assessed for purity using standard flow-cytometry technique. Purified monocytes were cultured in 6 well plates (TPP, Zollikofen, Switzerland), $2 \times 10^6$ cells/3 ml RPMI 1640 supplemented with 1% L-Glutamine, 1% antibiotics mix (Penstrep, Biological Industries, Bet-Haemek, Israel), 5% autologous serum, GM-CSF (200 units/ml) and IL-4 (500 units/ml) (R&D Systems, Minneapolis, USA), for 7 days. Half feeding with cytokines was performed every two days. The DCs collected from these cultures following the induction with the cytokine mixture, were further analyzed by flow-cytometry using markers characteristic of DC (see below).

*Preparation of DC-leukemia cell hybrids*

Dendritic – leukemia cell hybrids were generated by fusion as described by [20] using the chemical membrane-destabilizing polyethylene glycol (PEG, Roche Diagnostics, Mannheim, Germany). Cells were mixed at 1:1 ratio in 2 ml serum free RPMI 1640 in the presence of 50% PEG, for 5 min at room temperature. Cells were washed in serum free medium, resuspended in culture medium composed of RPMI 1640 supplemented with 10% heat inactivated FCS, 1% L-Glutamine, 1% antibiotics mix, 500 u/ml GM-CSF and cultured for 7 days in 6 well plates.

*Maturation of DC-leukemia hybrid*

Fused DC-tumor cells were subjected to maturation using a mixture of cytokines: TNF-α (100 ng/ml), IL-6 (1 μg/ml), IL-1β (50 ng/ml) (Peprotech, New Jersey, USA) and PGE2 (1 μg/ml, Cayman Chemical, Michigan, USA) for 3 days in culture medium. Maturation was assessed by flow-cytometry using the specific mature DC marker CD83 (Becton Dickinson, San Jose, CA, USA). After maturation, the fused cells were irradiated (3000 cGy) and used as stimulators for the generation of the specific CTLs. In addition dendritic cells alone were cultured in the presence of the same cytokines and used as controls.

*Phenotype analysis*

$2 \times 10^5$ cells were used for phenotypic analysis using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against CD86, major histocompatibility complex (MHC) class I, II, and phycoerythrin (PE)-conjugated mAbs against CD14 (DAKO, Glostrup Denmark) and CD83 (Becton Dickinson, San Jose, CA, USA). For tumor cells and DC-tumor hybrids analysis and CTL characterization, cells were stained respectively

with mAbs against CD15, CD13 (DAKO) and CD34 (Becton Dickinson, San Jose, CA, USA), or with anti CD3, CD4 and CD8 (DAKO). Cells were incubated with antibodies for 30 min at 4°C, washed in phosphate-buffered saline (PBS, Bet Haemek) and resuspended in 300 l PBS/sodium azide (0.02%, Sigma Aldrich, Steinheim, Germany) for analysis using the FACSCalibur flow-cytometer system (Becton Dickinson, San Jose, CA, USA). Appropriate isotype-matched control mAbs were also used.

*Fusion analysis*

*Fluorescent dye staining*

The immature DCs and leukemic cells were labeled with membrane fluorescent dye PKH 67 (green fluorophore) and fluorescent dye PKH 26 (red fluorophore) respectively, purchased from Sigma (Aldrich, Steinheim, Germany). The two cell populations were then fused as detailed above. After fusion, the cells were assessed for fusion efficiency by flow-cytometry. Double positive cells were recorded as hybrids. In addition, fluorescence microscopy analysis was performed on stained DC and tumor cells, as well as on the hybrids. The appearance of yellow fluorescence was characteristic of fused cells (Olympus BX52 fluorescence microscope equipped with green, red and Tric filters).

*Cytogenetic FISH analysis*

FISH analysis was performed with a Vysis® LSI® BCR/ABL Dual Color Translocation Probe (VYSIS, Downers Grove, Illinois U.S.A.). The BCR/ABL probe is a mixture of the LSI ABL probe labeled with SpectrumOrange and a BCR probe labeled with spectrumGreen. FISH was performed according to the manufacturer's instructions (VYSIS Inc.).

*Generation of CTLs*

Donor peripheral blood lymphocytes were purified using FH gradient and added to irradiated DCs-leukemia hybrids at a ratio of 3:1.

As a control, the lymphocytes were co-cultured with the irradiated unfused DCs and irradiated tumor cells alone, for 21-24 days. After one week of co-culture and every two days thereafter recombinant IL-2 (20 u/ml) (R&D Systems, Inc. Minneapolis, USA) was added to all cultures. In addition, same amount of stimulatory tumor cells, DCs and hybrids were added once a week.

*CTL activity and specificity assessment*

CTLs derived from the different co-cultures as well as unstimulated donor PBLs were tested for their cytotoxicity against the patient leukemic cells or third party specificity control tumor cells, using the Lacto-dehydrogenase (LDH) cytotoxicity assay (LDH Cytotoxicity Detection Kit, Roche, Penzberg, Germany) in several effector:target cell ratios.

Briefly, $5 \times 10^4$ tumor cells were incubated in a 96-well flat bottom plates with the effectors at different ratios for 4 hours at 37°C 5% $CO_2$. Then, the cells were processed as instructed by the manufacturer. Controls included medium, CTLs and tumor target cells for spontaneous and maximal release of LDH.

## 3. Results

Peripheral blood or bone marrow samples containing at least 90% leukemic cells were obtained from newly diagnosed AML and ALL patients. Tumor cells were detected primarily as an abnormal population upon CD45-side scatter FACS analysis. The gated tumor population was further analyzed for specific membrane markers such as CD15, CD7, CD19, CD34, and CD13, prior to freezing.

Peripheral mononuclear cells (MNC) from the prospective HLA-matched (A, B, C, DR and DQ) donors were frozen prior to any transplantation-related manipulation. These cells were used both a source of dendritic cells and of future CTLs.

### 3.1. Isolation and Purification of DCs

CD14 positive monocytes, positively selected by immunomagnetic beads from the mononuclear cells of the prospective selected donor, were cultured in the presence of human IL-4 and GM-CSF for 7 days. The immature DC's thus generated, were analyzed by flow cytometry for specific membrane markers. Results shown in **Figure 1**, demonstrate one representative experiment in which the membrane markers typical to immature DC's, *i.e.*, high expression of CD1a, CD86 and HLA, together with lack of CD14 and CD83 was evident.

### 3.2. Hybrids Generation

The tumor cells were thawed and assessed for viability and number as detailed in M & M.

Next, tumor cells and donor immature DC were mixed at 1:1 ratio in the presence of PEG for 5 min. Prior to mixing, a small number of cells were stained with membrane fluorescent dyes, the DC in green (PKH2) and the tumor cells in red (PKH26), in order to assess proper fusion, as detailed below. Fusion of these cells was performed separately, under same conditions.

Cells were then washed and cultured for 7 days in the presence of GM-CSF. Fusion efficacy was assessed according to several parameters.

*Validation of fusion*

Detailed below are several methods utilized to confirm the fusion of the dendritic and leukemia cells.

*Flow cytometry*

Double staining of membrane fluorescent dye, PKH2 and PKH26 by flow cytometry: A representative experiment is shown in **Figure 2(a)** in which about 35% of the analyzed cells were found to express both colors. In

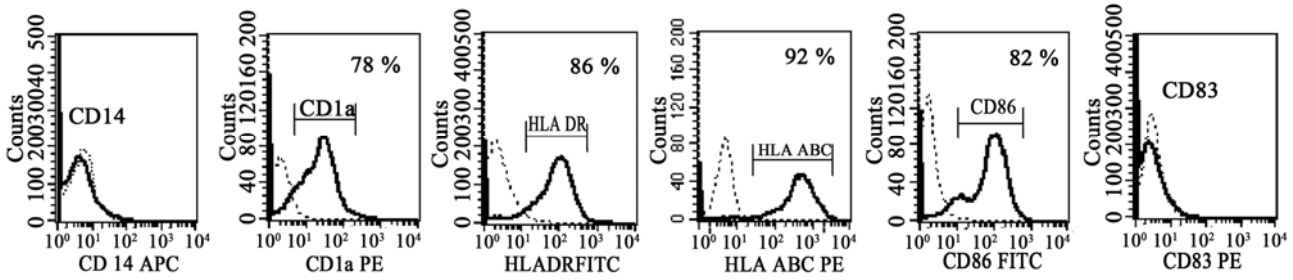



**Figure 1. Phenotype analysis of dendritic cells: FACS analysis of dendritic cell surface markers using specific monoclonal antibodies (bold line) and isotype controls (dot line). Immature dendritic cells obtained following cytokine induction for 7 days are shown. Histograms demonstrate negative expression of CD14 and CD83 and positive expression of CD1a, CD86, HLA class I and II.**

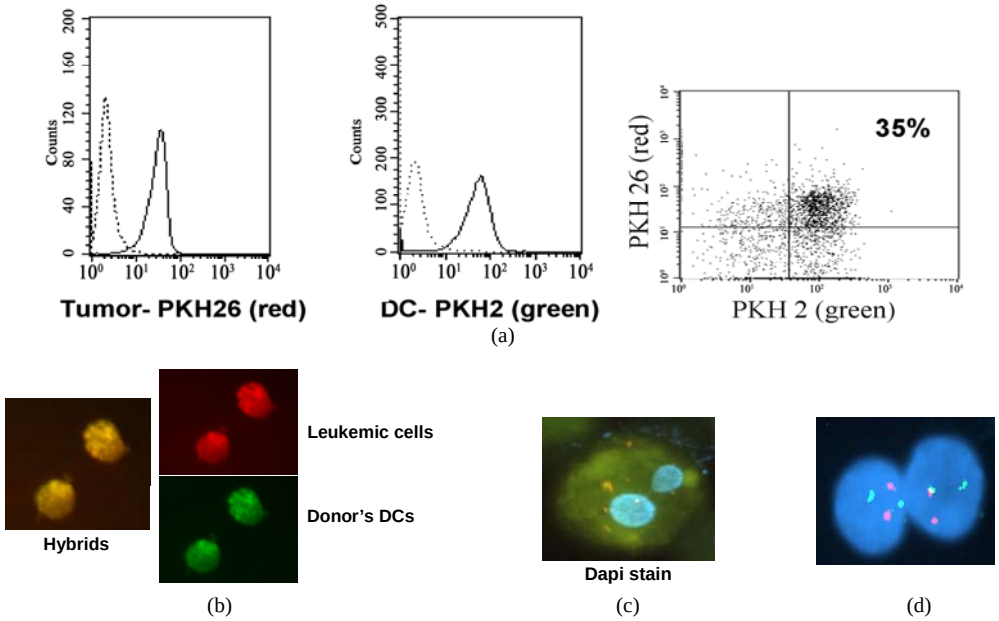



**Figure 2. Validation of fusion between leukemic cells and normal HLA matched DCs. (a) Assessment of fusion by immunofluorescent membrane dye. Leukemic cells from a Ph$^+$ ALL patient stained with PKH26 red fluorophore (left histogram) and DCs from his matched donor stained with PKH2 green fluorophore (right histogram). Bold lines represent the stained cells; dot lines histograms represent unstained cells. Dot plot analysis on the right panel shows double positive PEG induced hybrids. As shown, 35% of the cells in the mixed culture consisted of dendritic-tumor hybrids. Comparable results were obtained using AML cells; (b) Fluorescence microscopy of the DC-tumor hybrid cells: Microscope (Olympus BX52 fluorescence microscope) picture of cells from Figure 2(a) above. Same field was analyzed under 3 different filters (green, red and TRITC U-M61002). Upper right: red filter showing PKH26 stained tumor membrane, lower right: green filter showing PKH2 stained dendritic cell membrane. Left picture: TRITC filter showing the yellow combination of the hybrid membraneIdentical results were obtained using AML and ALL cells; (c) Fluorescence microscopy of an ALL-DC hybrid. Figure 2(c) demonstrates one cytoplasm containing two different nuclei visualized by Dapi stain. Identical results were obtained with AML cells. (d) FISH analysis of Ph$^+$ ALL-DC fused cells: Chromosome 9 specific ABL probe (red-orange fluorescence) and chromosome 22 BCR specific probe (green fluorescence) were used in order to visualize the chromosomes and the 9; 22 translocation (yellow fluorescence). Note normal cytogenetic picture on the left nucleus showing two 9 and two 22 chromosomes, while the right nucleus demonstrates the yellow 9; 22 translocation and one separate chromosome 9 and one separate chromosome 22.**

about five consecutive experiments an average of 36% of the cells were found to be double positive as statistically expected from a 1:1 (DC-tumor) ratio. A fluorescence microscopy of the fused cells was also conducted. This is shown in **Figure 2(b)**, in which the red and green fluorescence are demonstrated to yield a yellow light emission in presumably fused hybrid cells.

The tumor-fused DC cells were induced to mature using a cocktail of TNF, IL1-, IL-6, PGE2, GM-CSF and IL-4, for 2 days. The maturation of the dendritic tumor

fused cells was validated by the appearance of CD83. Occasionally, the co-expression of CD83 and of a tumor aberrant membrane marker such as CD15 or CD13 was also monitored by FACS analysis (data not shown).

*Cytogenetic FISH analysis*

Cells from the fused cultures were applied on microscope slides and stained with DAPI in order to visualize the nuclei. Numerous cells containing two nuclei were readily detected in the cultures as shown in **Figure 2(c)**. These experiments were performed with both ALL and AML cells with comparable efficiency and success. In order to identify the origin of the nuclei in the fused cells other slides were processed for FISH analysis as described above. Note that cytoplasm can not be visualized under the FISH staining protocol.

FISH based cytogenetic analysis was performed making use of the BCR-ABL translocation in $Ph^+$ ALL patients. This is demonstrated in **Figure 2(d)**. The picture demonstrates a fused cell that contained two nuclei: one nucleus containing two chromosomes 9 (red color) and 2 chromosomes 22 (green color); the second nucleus containing one chromosome 9, one chromosome 22 and a translocated 9-22 chromosome (yellow color).

### 3.3. CTL Generation

At the end of the maturation process, the tumor-DC fused cells were co-cultured with the donor mononuclear cells at a ratio of 10:1 MNCs: DC-tumor hybrid cells for 21-24 days. As controls, donor's mononuclear cells were also co cultured with autologous mature DC's alone and with the tumor cells alone (same tumor used for the fusion).

At the end of the incubation period, educated mononuclear cells were collected, washed and analyzed for cell phenotype. Cells were stained for membrane expression of CD3, CD4, and CD8. As shown in the left panel of **Figure 3** (representative experiment), most of the CD3 positive lymphocytes did not survive in the DC and tumor cultures and only about 20-30% of those cultures were CD3 positive cells. It should be noted that in these two groups, cell viability was very poor in comparison to Mononuclear cells were collected and stained for CD3-APC, CD4-PE and CD8-FITC and appropriate isotype

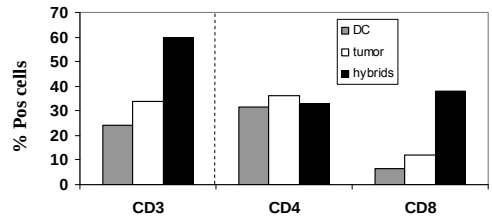



**Figure 3. Phenotype analysis of mononuclear cells co-cultured with dendritic cells, AML leukemic cells or DC-tumor hybrids for 21 days.**

controls. Cells were analyzed by FACS immunofluorescence. Bars represent the ratio of CD3 positive cells from the total number of cells in the cultures (left) and the percent of CD4 and CD8 positive cells from the CD3 positive ones in the various groups (middle and right).the hybrid group. Moreover, in the hybrid group 60% of the cells were CD3 positive and in this co-culture, a preferential induction of CD3 CD8 double positive cells was observed whereas CD3 positive CD4 positive cells were found in equal proportion in the three groups.

As shown, in the DC and tumor groups, most of the CD3 positive cells detected were not CD8 positive cells Thus, CD3 CD8 double positive cells, characteristic to CTL phenotype, were generated exclusively in co-cultures of donors mononuclear cells with the tumor-donor DC hybrids.

### 3.4. Cytotoxicity Assays

The cultured donor CTLs were assayed for cytotoxicity against their relevant AML patient target cells. In preliminary experiments, the ratio of co-culture was calibrated. Educated mononuclear cells were cultured with the tumor cells at various ratios. The cytotoxicity was assessed by LDH release as shown in **Figure 4(a)**. The results demonstrate clearly that the education of the CTLs on the hybrids generated the highest cytotoxic effect and that the optimal killing was observed at the 50:1 ratio. This ratio was further used in all coming experiments.

**Figure 4(b)** demonstrates the percent of tumor cells (3 AML and 1 ALL patients) killed by the educated CTLs in four consecutive experiments, using four different donors and their matched HLA recipient pairs. Up to 32% of the tumor cells were found to be killed by the CTLs educated on the hybrids as opposed to only 0-15% or 1-2% in the controls.

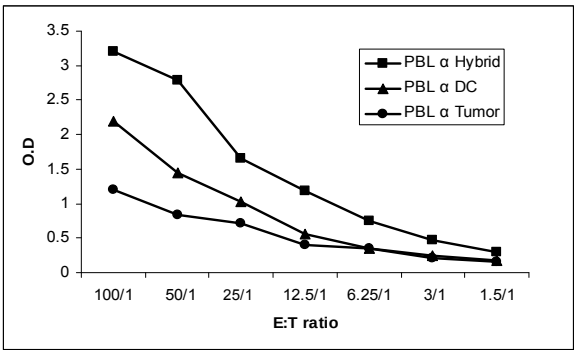
A limited specificity study was initiated with the use of an unrelated tumor of the same histological type (AML). This is shown in **Figure 5** in two consecutive experiments. The results clearly demonstrate that the CTLs generated against the relevant tumor cell-DC hybrids were not cross reacting against irrelevant, unrelated leukemia cells. In addition, no cytotoxicity could be detected in the mononuclear cell population prior to education on the DC-tumor hybrids.

## 4. Discussion

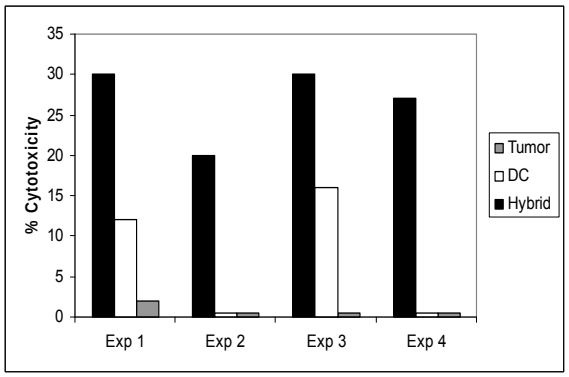
The primary massage of this communication concerns the proof of concept related to the in vitro generation of tumor specific CTLs educated on DC-tumor hybrids in an HLA matched setting. This has never been attempted before.

The therapeutic options for patients that relapse with




(a)




(b)

**Figure 4. Cytotoxic activity of HLA-matched donor CTLs. (a) Calibration of LDH release for optimal E:T ratio: CTLs of the donor (originally PBLs) generated against DC, tumor cells or DC-tumor hybrids were assayed for cytotoxicity against tumor cells upon incubation for 4 hours in 96 well plates at 37°C. At the end of the incubation, cultures were processed as instructed by the manufacturer of the LDH Cytotoxicity Detection Kit. Lines represent O.D. of LDH released at the different ratios in the different groups; (b) Anti tumor cytotoxicity of HLA-matched CTLs: Four separate cytotoxicity assays using four different donor-patient sets at 50:1 E:T ratio, were performed. The killing of the tumor target cells by the CTLs generated on DC, tumor, DC-tumor hybrids was calculated specifically as instructed. Bars represent the percent of tumor cells killed by CTLs in the various groups.**

leukemia after allogeneic stem cell transplantation are limited and their overall prognosis is poor. At an early stage of the relapse, cell-mediated immunotherapy with donor lymphocytes (DLI), aimed to induce a potent graft-versus-leukemia (GVL) effect, is most commonly used [25,28,29,35-37]. This methodology however, has certain significant drawbacks. First, the immune cells injected are naïve and therefore are expected to undergo tumor priming *in vivo*. This process may not be sufficiently efficient and the leukemic process may outgrow the GVL effects. Second, the *in vivo* priming may not be restricted to the tumor antigens only. Indeed, an undesirable graft versus host (GVH) response is often observed after DLI [38,39]. Third, the donor lymphocytes infused, not being properly manipulated, may also contain regulatory, suppressor cells [40]. Several investigators have indeed tried to manipulate the DLI prior to its infusion for better and more specific anti-leukemic effect. For example, depletion of CD8 positive cells [41,42], activation of donor T cells with anti-CD3 and anti-CD28 coated beads [43], *ex vivo* insertion of suicide genes into donor T cells prior to DLI [44]. Others have used donor NK cells alloreactivity in matched and mismatched hematopoietic transplants [45,46]. Accordingly the main concern of the present study is the development of an additional, improved methodology for the use of DLI immunotherapy.

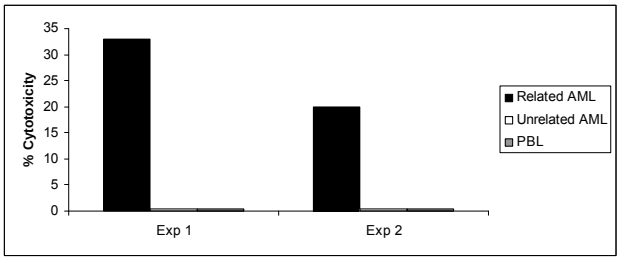



**Figure 5. Specificity of hybrid-stimulated CTLs. CTLs were generated on DC-AML hybrids and assayed for cytotoxicity on the same AML target cells used for the hybrid generation, or AML cells derived from a different patient (HLA unmatched). Unstimulated PBLs (designated in the figure as PBL) from the same donors were assayed for cytotoxicity in the same experiments. Two different donor-patient experiments are shown. (Cytotoxic assay as described in legend to Figure 4).**

Administration of autologous or allogeneic cytotoxic T lymphocytes, generated by co-stimulation with dendritic-tumor cell hybrids, tailored to present the putative tumor specific antigens for each individual patient, was already proven to be effective in other malignancies such as prostate cancer and melanoma, as well as in the treatment of post-transplant CMV infections [47-49].

Tumor-specific DLIs are likely to be the most effective method of immunotherapy to achieve a maximal GVL activity. The aim of our study was to induce in vitro a potent donor anti-leukemia cytotoxic T lymphocyte (CTL) response, utilizing HLA-matched dendritic-leukemia cell hybrids. These leukemia-specific donor CTLs are intended to treat patients with post-transplant relapsed leukemia, after HLA-matched allogeneic stem cell transplantation.

Our results show that such in vitro response is indeed attainable, and that the DC-tumor fusion approach is feasible when the CTLs and the tumor are HLA (A, B, C, DR, DQ) matched.

Elicitation of CTLs across HLA-matched partners was also demonstrated using DC-tumor cell mixing in vitro without fusion [15,17] or even directly by exposing donor CD8 cells to patient leukemic cells [50]. Nevertheless, it has been strongly suggested by numerous groups that methodology using hybrid cell generation of DCs and tumor cells is more effective in treating malignancies and even metastatic cancer [5,20,51-54].

The stimulatory antigens as well as the target antigens responsible for the CTL induction and activity shown here are largely unknown. We presume that these are tumor specific entities but the involvement of minor histocompatibility antigens can not be excluded. For therapeutic purposes it will be vital to identify the specific CTL clones directed against tumor antigens and "normal" antigens, for example expressed by fibroblasts of the same patient as also suggested by Montagna and Falkenburg [17,50,55]. Characterization of such clones could be then followed by an isolation and expansion of the appropriate CTLs to be injected to patients.

The other alternative would be to administer the DC-tumor cell hybrids with or without the "classical" naïve DLI. Injection of such hybrids has already proven effective in certain solid tumors, although in these cases the DCs were either autologous or derived from a third party donor [56] to the tumor.

The validation process of the fusion efficiency was achieved by a number of methodologies to ensure that the PEG mediated fusion generated the appropriate cell composition. This is particularly important since cell aggregates and not genuine fused cells are sometimes induced in the absent of PEG.

It should be noted however that the only way to maximize the probability to obtain the appropriate heterofusion (that is a dendritic cell and a tumor cell) is to start with a pure tumor cell population, although irrelevant fusions of cells cannot be prevented completely. In that respect, Shu *et al.* have recently formulated a provocative statement about the lack of evidence for heterokaryonic fusion cell formation in various clinical trials and publications. They claim that the evidence for such cell formation was not definitive and this fact resulted maybe in a misconception about the low efficacy of fusion hybrid vaccine. They believe that conclusions drawn from reported clinical trials have not properly evaluated the efficacy of the vaccine and therefore they neither confirm nor disclaim the potential benefits that may be derived from this form of immunotherapy [57].

The CTL activity itself, as assessed by the LDH release assay, was found to be effective and specific, based on a number of experiments performed. It should be noted that we obtained about 30% killing of the tumor cells. We think that this represents an underestimate of the actual number of the tumor cell eliminated by the CTLs because the LDH assay might not be specific enough, due to the spontaneous release of LDH by the effector cells themselves, especially when high numbers are employed.

In some of the experiments a low but significant rate of tumor cell killing was obtained following the education of CTLs on dendritic cells alone. We speculate that this could be due to the induction of NK activity in the PBL's exposed to autologous dendritic cells. We cannot exclude at this point that the direct exposure of lymphocytes to autologous DC's, may lead to the induction of low non-specific cytotoxic activity. It should be noted however, that the exposure of the same lymphocytes to the tumor cells only, in the same experiments, did not lead to any cytotoxic effect. In this respect, as shown here the co-culture of PBLs with DCs or with tumor cells did not result in the induction of CD8 positive cells as opposed to the high CD8 generation following the co-culture of PBLs with the hybrids. No preferential generation of CD4 cells was observed.

Our primary objective was to develop an improved DLI source for active and specific allogeneic cell immunotherapy for post-transplant leukemic relapse in an HLA matched setting. The technology needs to be further developed but the *in vitro* feasibility demonstrated in this study seems promising.

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# Management of Unsuspected Gallbladder Cancer in the Era of Minimally Invasive Surgery

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## ABSTRACT

*Gallbladder cancer is the most common malignant tumor of the biliary tract and often diagnosed incidentally during laparoscopic cholecystectomy for gallbladder disease. This underscores the importance of considering the diagnosis of gallbladder cancer when confronted with atypical findings and understanding cancer management guidelines that may impact patient outcome. Potentially curable early stage gallbladder lesions are often missed during preoperative workup and discovered incidentally during difficult cholecystectomy or afterwards on pathologic diagnosis. We will review current strategies to diagnose early stage disease and discuss clinical management issues to optimize the outcome of patients with gallbladder cancer.*

***Keywords*: *Gallbladder Cancer, Laparoscopic Cholecystectomy, Radiologic Imaging, Surgical Management***

## 1. Introduction

Gallbladder cancer is the most common malignant tumor of the biliary tract and early-stage disease is typically diagnosed incidentally with the widespread adoption of laparoscopic cholecystectomy for symptomatic gallbladder disease. It has been estimated that approximately 1% of elective cholecystectomies will result in an unexpected diagnosis of gallbladder cancer [1,2]. As laparoscopic cholecystectomy represents one of the most common general surgery procedures performed in the United States with over 700,000 cases each year [3], it underscores the importance that all surgeons and practitioners consider a differential diagnosis of gallbladder cancer when confronted with atypical findings and be familiar with cancer management guidelines that directly impact patient outcome. Since advanced gallbladder cancer is associated with a uniformly dismal prognosis, we view the diagnosis of early-stage disease as a critical step in clinical management. It is important to maintain a certain level of suspicion in all patients presenting with right upper quadrant complaints. Potential curable early stage gallbladder lesions are often missed during preoperative workup and discovered incidentally during difficult cholecystectomy or afterwards on pathologic diagnosis. We will review current strategies to diagnose early gallbladder cancer and discuss clinical management issues to optimize the outcome of patients with gallbladder cancer.

## 2. Incidence and Epidemiology

Cancers of the extrahepatic biliary tract are the sixth most common gastrointestinal malignancy diagnosed in the U.S. exceeding 9000 cases per year [4]. Gallbladder cancer, typically a highly aggressive and lethal malignancy, represents nearly two-thirds of these cases. Rates of gallbladder cancer vary widely by geographical location. Native North and South American populations report the highest rates of disease, while gallbladder cancer is rarely seen among Caucasian populations in Western Europe and the United States. Additionally, incidence rates within the United States are noted to be higher among certain ethnic populations such as Native and Hispanic Americans [5,6]. This geographic and ethnic distribution closely parallels the epidemiology of gallstone disease, which is considered an important risk factor for the development of gallbladder cancer [7]. Gallbladder carcinoma is also more common in females by nearly a 3:1 ratio compared to men, and incidence increases with advancing age [5].

While no definitive cause has been identified for the development of cancer of the gallbladder, several eti-

 

ologic risk factors have been described. Cholelithiasis has long been associated with the development of gallbladder cancer [7-9] with gallstones reported in over 90% of patients with gallbladder cancer. The epidemiologic profiles of gallstone disease and gallbladder cancer are similar, with higher rates observed among women, elderly, and certain ethnic groups. Chronic cholecystitis and porcelain gallbladder have also been associated with an increased incidence of gallbladder cancer [10-12]. The chronic inflammatory state associated with both conditions has been proposed as an important factor promoting the development of mucosal dysplastic changes that progresses to invasive carcinoma [5]. Other reported risk factors for the development of gallbladder cancer include the presence of choledochal cysts, adenomatous gallbladder polyps, obesity, tobacco smoking and various occupational chemical exposures including azotoluene and nitrosamine [5,13].

## 3. Diagnosis

Early suspicion and diagnosis of gallbladder cancer is one of the most challenging aspects of clinical management since presentation can be highly variable. Patient complaints such as intermittent right upper quadrant pain, fever, or shoulder pain are more often attributed to cholelithiasis and cholecystitis rather than an underlying malignancy. Patients who present with jaundice, weight loss, or persistent right upper quadrant pain from gallbladder cancer are usually found to have locally advanced or metastatic disease, and are often not suitable candidates for curative resection. Preoperative diagnosis of early stage disease is often difficult secondary to low clinical suspicion, lack of specific presenting signs and symptoms, and equivocal imaging findings. Yet, it is at this stage that the diagnosis is most important as it is in this stage that adequate planning for the operation will have significant impact on patient survival. Certain findings on imaging studies should raise the suspicion of a patient at risk of an underlying gallbladder cancer. Mass lesions, gallbladder polyps, and porcelain gallbladder can easily be identified by standard imaging studies used to workup gallbladder disease and warrant specific attention.

Ultrasound is usually the first imaging study obtained in the workup of suspected gallbladder pathology because of its low cost, widespread availability and accuracy in diagnosing gallstones/sludge and other types of gallbladder disease. In spite of its high sensitivity for the detection of tumor in advanced stages, it is limited in the diagnosis of early lesions and is unreliable for staging. Gallbladder carcinoma may appear as a mass completely occupying or replacing the gallbladder lumen, but can also have a more subtle appearance such as focal or diffuse asymmetric wall thickening or as an intraluminal polyp (**Figure 1**) [14]. A mass occupying or replacing the gallbladder lumen is the most common pattern seen in 40-65% of the patients at initial detection. Direct invasion of the surrounding liver parenchyma is highly suggestive of gallbladder carcinoma [14,15]. On ultrasound, the tumor usually appears hypoechoic. However, necrotic tumor or trapped bile may be anechoic. Furthermore, echogenic shadowing foci from gall stones, porcelain gallbladder, or tumoral calcification may also be present [16].

Focal or diffuse asymmetric wall thickening is seen in 20-30% of the cases. The differential diagnosis for gallbladder wall thickening is wide and includes acute and chronic cholecystitis, xanthogranulomatous cholecystitis, and adenomyomatosis. Systemic diseases such as acute hepatitis, portal hypertension, and congestive cardiac failure can also present with gallbladder wall thickening [15,17]. Asymmetric, irregular, or extensive wall thickening with marked arterial phase enhancement that persists or becomes isodense or hypointense to the liver in the portal venous phase should heighten the suspicion of gallbladder carcinoma (**Figure 2**) [18,19].

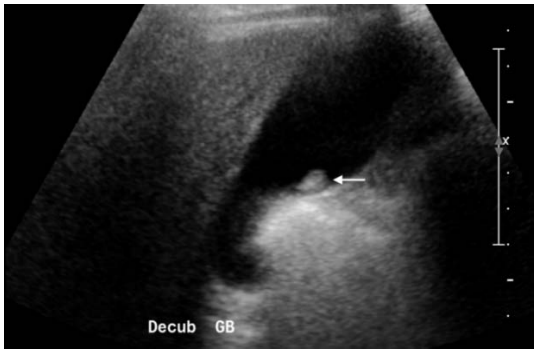


**Figure 1. US image of an immobile echogenic focus arising from the gallbladder wall consistent with a gallbladder polyp (arrow).**

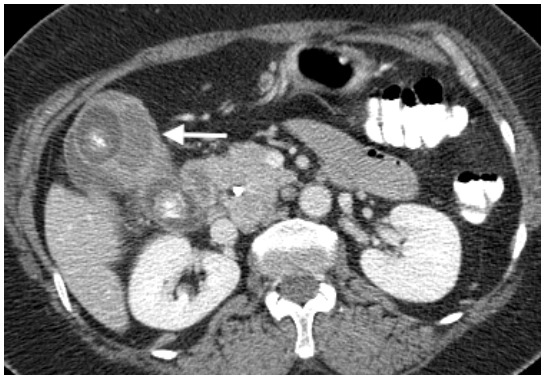

**Figure 2. Contrast-enhanced CT scan axial image of a patient with gallbladder carcinoma. Soft tissue enhancing mass is seen in the gallbladder lumen (arrow). Two gall stones are also present.**

 

Polyps are immobile echogenic foci arising from the gallbladder wall (**Figure 1**). At the time of ultrasound, if there is notable movement of a polypoidal mass, biliary sludge or clot can be diagnosed. Malignant lesions of the gallbladder present as a polypoidal lesion in 15-25% of cases and are usually larger than 1 cm with a thickened implantation base. Polypoid lesions of less than 1 cm are rarely cancer [20]. The differential diagnosis of a polypoid gallbladder lesion includes adenomatous or hyperplastic cholesterol polyps as well as uncommon tumors such as carcinoid or melanoma metastases [15]. Most radiologists typically recommend follow-up sonograms at 6- to 12-months intervals for polyps between 5 mm and 1 cm in size [21]. FDG-PET has a limited role in this setting and may result in false-positive interpretations [22].

CT and MRI are more widely used for the characterization of potentially malignant gallbladder lesions and to evaluate the extent of metastatic disease [15]. Primary gallbladder carcinoma is usually hypodense on unenhanced CT and enhances after IV contrast administration. On MRI, gallbladder carcinoma is usually hypo- to isointense on T1-weighted and moderately hyperintense on T2-weighted sequences. On both CT and MRI, intense irregular enhancement may occur at the periphery of larger lesions during the early arterial phase. Enhancement may be retained in the fibrous stromal components of the tumor in the portal venous and delayed phases, aiding differentiation from the classic hepatocellular carcinomas which have a greater tendency to washout [15,18]. Multidetector CT (MDCT) has a reported accuracy of 85% in the evaluation of the extent of local disease and in predicting respectability through its ability to delineate hepatic and vascular invasion, lymphadenopathy, and distant metastases [15,23,24]. Multiplanar and 3-D volume-rendered reconstructions are useful for surgical planning. MR cholangiopancreatography (MRCP) and 3-D MR angiography can reliably detect bile duct and vascular invasion, but are less sensitive for distinguishing hepatic invasion and lymph nodal metastases compared to CT [25].

PET imaging can demonstrate intense uptake of 18-F-FDG for malignant gallbladder tumors, but lacks the sensitivity in distinguishing primary gallbladder carcinoma from other malignant lesions that can invade the gallbladder fossa such as hepatocellular carcinoma, cholangiocarcinoma, or metastatic disease [15]. However, PET-CT has the potential to detect unsuspected metastases and alter staging and therapy [22,26]. Prospective studies directly comparing the efficacy of CT, MRI, and PET-CT to diagnose and stage gallbladder carcinoma have not been performed [15].

## 4. Staging

In spite of the timing of diagnosis in relation to definitive surgery, gallbladder cancer prognosis is dependent on staging as outlined by the American Joint Committee on Cancer TNM staging classification. Significant changes in the most recent 7th edition introduced in January 2010 include the designation of cystic duct involvement, distinction between involvement of regional hilar lymph nodes and distant regional nodes (celiac, periduodenal, peripancreatic, and superior mesenteric nodes), and a reclassification of unresectable locally advanced T4 tumors as stage IV disease (**Table 1**). Gallbladder cancer is a highly aggressive malignancy with disappointingly low rates of long term survival, especially for patients with locally advanced (T4), node positive (N1/2), and metastatic (M1) disease. Generally speaking, gallbladder cancer patients presenting with symptomatic disease are often found to have unresectable stage IV disease and typically palliated with conservative systemic therapy. Despite the aggressive nature of this disease, carefully selected patients with nodal metastases have been reported to undergo radical resection with observed 5-year survival rates between 26-45% [27-30].

## 5. Operative Management

In the absence of distant metastasis or nodal disease (stage 0-IIIA), surgery is the mainstay treatment for early

**Table 1. TNM staging classification for gallbladder cancer (AJCC, 7th Ed.).**

| | |
|---|---|
| **TX** | Primary tumor cannot be assessed |
| **T0** | No evidence of primary tumor |
| **Tis** | Carcinoma in situ |
| **T1a** | Tumor invades lamina propria |
| **T1b** | Tumor invades muscular layer |
| **T2** | Tumor invades perimuscular connective tissue |
| **T3** | Tumor perforates serosa or directly invades the liver and/or one other adjacent organ |
| **T4** | Tumor invades main portal vein or hepatic artery or invades multiple extrahepatic organs |
| **NX** | Regional nodes cannot be assessed |
| **N0** | No regional nodal metastasis |
| **N1** | Metastasis to nodes along the cystic duct, common bile duct, hepatic artery and/or portal vein |
| **N2** | Metastasis to periaortic, pericaval, superior mesenteric artery, and/or celiac artery lymph nodes* |
| **M0** | No distant metastasis |
| **M1** | Distant metastasis |

| | | | |
|---|---|---|---|
| **Stage 0** | Tis | N0 | M0 |
| **Stage I** | T1 | N0 | M0 |
| **Stage II** | T2 | N0 | M0 |
| **Stage IIIA** | T3 | N0 | M0 |
| **Stage IIIB** | T1-3 | N1 | M0 |
| **Stage IVA** | T4 | N0-1 | M0 |
| **Stage IVB** | Any T | N2* | M0 |
| | Any T | Any N | M1 |

* Denotes changes from 6th edition classification.

 

gallbladder cancer with the specific procedure primarily dictated by the T stage of disease. Tis and T1a tumors are adequately treated by simple cholecystectomy with expected 5-year survival rates between 90 to 100% [31-33]. Outcomes for more advanced T stage tumors treated by simple cholecystectomy are significantly worse. T1b and T2 tumors are best treated by radical cholecystectomy which consists of an extended gallbladder resection including partial liver segments IVB and V and portal lymphadenectomy, a procedure that should be performed in centers with high volume of extrahepatic bile duct and liver resections. In addition to radical cholecystectomy, T3 tumors may also necessitate en bloc resection of any involved adjacent viscera. Extrahepatic bile duct resection, more extensive hepatectomy, and combined with pancreaticoduodenectomy has been described for advanced cases with cystic duct or extrahepatic bile duct involvement with unclear survival benefit [28,34,35]. As was the case in patients who need a careful segment IVB and V resection, we recommend that these patients be treated in centers that specialize in complex hepatobiliary resections.

**Incidentally Discovered Early Stage Disease**

Most early stage tumors are unrecognized and patients frequently undergo laparoscopic cholecystectomy for presumed symptomatic cholelithiasis or acute cholecystitis. Despite advances in ultrasound and CT imaging, small tumors confined to the mucosa often escape detection with currently available imaging modalities. If there is any equivocal imaging or intraoperative findings, it is imperative that the resected gallbladder specimen be opened and carefully inspected by the primary surgeon to assess for any suspicious mucosal lesions. If they are found, the specimen should be evaluated by a surgical pathologist immediately. Even if the surgeon is unprepared to proceed with a more radical resection as indicated for T1b or greater tumors, establishment of a definitive pathologic diagnosis will expedite further staging workup and referral to an experienced hepatobiliary surgeon. It is of paramount importance for the primary surgeon to document any unintended events such as gallbladder injury with resultant bile or stone spillage, and to liberally utilize a specimen retrieval bag for gallbladder removal to minimize intraabdominal dissemination and wound or port site contamination.

The majority of T1 tumors are discovered following uneventful cholecystectomy on final pathologic analysis. Cure rates for invasive disease limited to the mucosa treated by simple cholecystectomy exceed 90% provided negative resection margins are confirmed. Cystic duct margin status should be carefully noted as a positive duct margin may necessitate more aggressive re-resection to achieve an R0 resection status, even if biliary reconstruction will be required. Controversy exists over the benefit of performing radical cholecystectomy in patients with T1b tumors invading the gallbladder muscular layer. Several recent series have demonstrated decreased survival for patients with T1b tumors treated by simple cholecystectomy alone [33,36-38]. Patients with T2 disease invading through the muscular wall whom are medically fit and without evidence of distant disease should be offered radical re-resection since recurrence rates can be expected to exceed 30% with cholecystectomy alone [36].

**Oncologic Impact of Laparoscopic Surgery**

The advent and widespread application of minimally invasive surgery has been shown to improve patient outcomes. Decreased patient morbidity, hospital length of stay, pain medication requirement, and return of bowel function are commonly associated with laparoscopic abdominal procedures with high patient satisfaction.

The use of minimally invasive surgery techniques in GI cancer patients is a controversial topic with limited evidence available to establish standard practice guidelines [39]. Though a well conducted randomized trial demonstrated oncologic equivalence for laparoscopic colectomy in the treatment of colon cancer [40,41], the efficacy of minimally invasive procedures for other GI malignancies remains unproven. There are specific technical aspects of gallbladder surgery that deserve particular attention including avoidance of bile contamination and port-site recurrence. Additionally, there have been reports of specific immunologic changes associated with abdominal insufflation (and circulation and re-circulation of $CO_2$) used during laparoscopic surgery [42]. Laparoscopic abdominal procedures in animal models have shown decreased cell-mediated immunity and stimulation of cytokine responses which may impact host-tumor responses. One human study reported elevated plasma levels of vascular endothelial growth factor (VEGF), a potent promoter of tumor vascular angiogenesis, following laparoscopic resection for colorectal cancer [43]. Further studies are needed to determine whether these described biologic and immunologic responses impact cancer prognosis in patients undergoing minimally invasive resection.

**Immediate Versus Delayed Radical Cholecystectomy**

Patients diagnosed with T1b, T2, and T3 tumors who do not have distant metastatic disease and who are otherwise fit for a major operation should be treated by an extended resection with curative intent. Radical cholecystectomy consists of cholecystectomy (if not previously performed), partial liver resection of segments IVB and V which contain the gallbladder fossa, combined with portal lymphadenectomy and extrahepatic bile duct resection when there is evidence of cystic duct or hepatic

 

duct involvement. The improved survival benefit of radical resection has been clearly demonstrated in numerous studies compared to simple cholecystectomy [6,44-46]. With the widespread adoption of laparoscopic cholecystectomy, increasing numbers of early stage gallbladder tumors are diagnosed postoperatively and concern exists whether prognosis is negatively impacted by delayed recognition and two-staged surgical approach. Intraoperative gallbladder perforation has been demonstrated to upstage and negatively impact survival even for patients with T1 lesions [3,47]. It is therefore prudent to maintain a low threshold for open cholecystectomy if preoperative imaging is suggestive of an underlying malignancy or conversion to an open procedure if intraoperative findings are suggestive of an invasive gallbladder tumor. Regardless of initial open or laparoscopic approach, Fong et al. and others have demonstrated equivalent outcomes following re-resection when compared to patients undergoing immediate definitive management [47-50]. These data indicate that a delayed surgical approach for definitive treatment, regardless of the initial cholecystectomy technique does not adversely affect outcome provided the integrity of the gallbladder wall is maintained.

**Management of Port-Site Contamination**

Advanced gallbladder cancer can present with peritoneal metastasis and port-site recurrences which has been reported in 2-28% of patients following laparoscopic cholecystectomy [33,51]. It should be noted that early studies reporting high port site recurrences were likely conducted prior to the routine use of gallbladder retrieval bags. Recent studies have demonstrated equivalent rates of wound recurrences when comparing open versus laparoscopic cholecystectomy for early gallbladder cancer [33]. Whether wound recurrences are the result of technical complications of cholecystectomy, biologic properties of laparoscopic insufflation or an indicator of tumor biology is unknown. Careful avoidance of gallbladder perforation and use of a retrieval bag are important measures to consider during difficult laparoscopic cholecystectomy and may be an indication for conversion to an open procedure. Though the effect on long term outcomes is debatable, we advocate routine port-site resection if there is evidence or suspicion of prior tumor contamination at the time of radical cholecystectomy.

**Treatment of Porcelain Gallbladder**

Porcelain gallbladder is a manifestation of chronic cholecystitis characterized by intramural calcification of the gallbladder wall presumably from longstanding inflammation (**Figure 3**). It is a rare finding, identified in only 0.06 to 0.8% of cholecystectomy specimens, and is associated with gallstones in 95% of patients [11,52]. Past studies have described a close association between

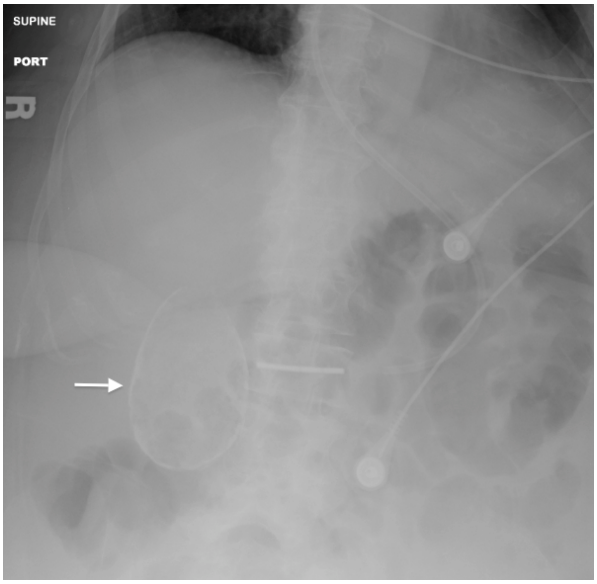


**Figure 3. Portable abdominal radiograph showing densely calcified wall (arrow) of the gallbladder consistent with a porcelain gallbladder.**

porcelain gallbladder and gallbladder cancer and have reported rates of incidental carcinoma in 12-61% of porcelain gallbladder specimens[52]. This has led many to advocate elective cholecystectomy for all patients found to have radiographic evidence of porcelain gallbladder even in the absence of symptoms. Recently, the validity of this association has been challenged. Two large retrospective series evaluated gallbladder specimens and found a significantly lower incidence of gallbladder cancer in calcified gallbladders, and suggested that the pattern of calcification influences the risk [11,52]. No patients in both studies who had diffuse, transmural calcifications were found to have a primary gallbladder malignancy, while the incidence of cancer in specimens with selective mucosal calcifications was 7% [11].

Patients diagnosed with porcelain gallbladder should undergo careful assessment. The risk of underlying cancer appears to be much lower than originally reported, but may be significant in specific situations such as those patients with selective mucosal calcifications. Management strategies should take into account patient preference, operative risk, and overall risk of underlying malignancy. If operative intervention is undertaken, porcelain gallbladder is not considered an absolute contraindication to laparoscopic cholecystectomy, provided strict avoidance of gallbladder perforation and use of a specimen removal device is employed with a low threshold to open conversion.

**Role for Lymphadenectomy**

Lymph node involvement in gallbladder cancer has

 

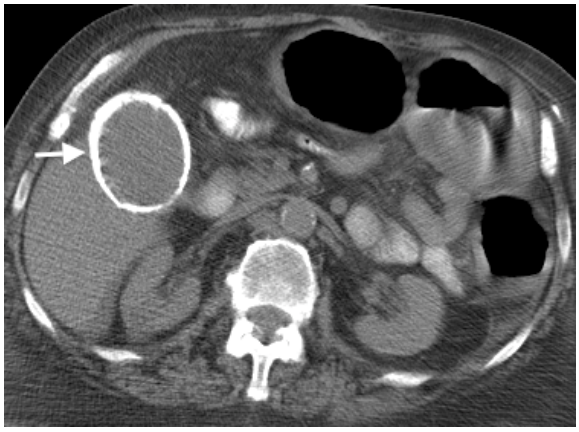

**Figure 4. Axial CT scan image of the same patient with porcelain gallbladder (arrow).**

been demonstrated to be a significant predictor of poor outcome [30,44,53]. The recently updated AJCC staging criteria distinguishes regional hilar nodal metastases (stage III) from peripancreatic, periduodenal, celiac and SMA locations which are considered as distant metastatic disease (stage IV). Increasing T stage correlates with the risk of nodal metastases. T1 tumors are associated with a low risk of lymph node involvement between 0-12% [53,54]. However, T2 and T3 tumors have been shown to have a 31% and 46% incidence of nodal metastases, respectively [54]. The prognosis of patients with regional nodal metastases (N1) is poor with one well-established hepatobiliary center reporting a median survival of 18 months and 5-year survival of 17% in patient undergoing definitive resection [28]. Distant nodal metastases (N2) should be considered a contraindication for radical resection given that the outcomes of these patients are uniformly poor. In the absence of distant metastatic disease, including N2 disease, we advocate the inclusion of hilar nodal dissection during radical cholecystectomy to complete pathologic staging, provide important prognostic information, and achieve a negative margin (R0) resection.

**Bile Duct Resection and Extended Hepatic Resection**

Proximal involvement of the cystic duct margin may benefit from extended hepatic parenchymal or extrahepatic bile duct resection. Careful pathologic assessment of the cystic duct margin following simple cholecystectomy is required when planning potential radical re-resection. Intraoperative evaluation with liberal use of ultrasound and frozen section analysis are useful adjuncts to adequately determine extent of potentially curative resection. While routine extrahepatic biliary resection has not been shown to provide additional survival benefit in the setting of a previously documented negative cystic duct margin, more extensive en bloc hepatic resection is recommended for locally advanced disease as an R0 resection offers the only chance for long term cure [28,54].

**Adjuvant Therapy**

The role for adjuvant radiation and chemotherapy in the treatment of gallbladder cancer is unclear. While several studies have demonstrated modest survival benefits, numbers are small and randomized data is lacking. One study showed an improved 5 year survival of 26% for patients treated with 5-fluoruracil and mitomycin C compared to a control group (14%), however not all patients underwent complete R0 resection confounding interpretation of these results [55]. Currently, we do not recommend the routine use of adjuvant therapy following adequate definitive resection of gallbladder cancer outside a clinical trial setting.

## 6. Summary

Gallbladder cancer is a highly aggressive malignancy which is difficult to diagnose in its early, curative stages. Consequently, late diagnosis yields poor outcomes. In the era of minimally invasive surgery and widespread practice of laparoscopic cholecystectomy for gallbladder disease, an opportunity exists to diagnose patients with gallbladder cancer at potentially curable early stages. A reasonable suspicion when common diagnostic modalities show suspicious signs should lead to a more complete work-up. This, in turn will facilitate appropriate operative planning for the surgeon. A proper understanding of the principles of treatment for gallbladder cancer is of paramount importance to improve survival for patients diagnosed with incidental gallbladder cancer. Every attempt should be made to avoid unnecessary surgery that may increase the risk of cancer dissemination. Definitive management of this challenging disease should be referred to an experienced multidisciplinary hepatobiliary center to offer patients the greatest opportunity for long term survival

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# A Case of Advanced Multiple Hepatocellular Carcinomas with Portal Vein Tumor Thrombosis Successfully Treated by Oral Tegafur/Uracil

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## ABSTRACT

*A case of advanced multiple hepatocellular carcinomas* (*HCC*) *with portal vein tumor thrombosis successfully treated by oral tegafur/uracil is reported. A* 69*-year-old Japanese woman with advanced HCC with tumor thrombosis underwent transcatheter arterial infusion chemotherapy in April* 2001*. However,* 1 *year later, the patient experieced a recurrence with advanced multiple HCC with portal vein tumor thrombosis and ascites. Treatment with oral tegafur/uracil was started in May* 2002 *and resulted in the partial response of liver tumors and the complete improvement of ascites. She remained in good health for about* 6 *years. This case strongly suggests that oral tegafur/uracil is an effective treatment for some cases of advanced HCC with portal vein tumor thrombosis.*

***Keywords*****:** *Advanced Hepatocellular Carcinoma, Portal Vein Thrombosis, Oral Tegafur/Uracil, Chemotherapy*

## 1. Introduction

Various treatment modalities, such as transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection therapy (PEIT), and radiofrequency ablation (RFA) have been recently developed and are used worldwide to treat patients with hepatocellular carcinoma (HCC) [1-4]. However, the use of these modalities for advanced HCC is limited.

On the other hand, it has been reported that patients with multiple HCCs with tumor thrombosis in the major portal branches were successfully treated with intra-arterial 5-fluorouracil perfusion chemotherapy combined with subcutaneous interferon-alpha administration [5,6]. Such combination therapy may be a promising treatment modality for advanced HCC with tumor thrombosis. However, the prognosis of advanced HCC with tumor thrombosis (VP4) is still unsatisfactory. A rare case of advanced multiple HCCs with portal vein thrombosis (VP4) that was successfully treated by oral tegafur/uracil (UFT®, Taiho Pharmaceutical Co. Ltd. Tokyo, Japan) and had a good prognosis, with survival for more than 5 years, is reported.

## 2. Case Presentation

A 69-year-old woman with advanced HCC with tumor thrombosis underwent transcatheter arterial infusion chemotherapy in April 2001. However, 1 year later, the patient experienced advanced multiple HCCs with portal vein thrombosis (VP4) and ascites (**Figure 1**). As shown in **Table 1**, the patient had decompensated liver cirrhosis due to hepatitis C virus. On admission, the alpha-fetoprotein (AFP) level was 317.2 ng/ml, and the level of tumor marker known as protein-induced vitamin K antagonist II (PIVKA-II) was 162,210 mAU/ml. Treatment, such as TACE, PEIT, or RFA, was not administered. She was treated with oral UFT® in May 2002. This treatment resulted in the partial response of liver tumors and the complete improvement of ascites (**Figure 2**). At the same time, the AFP level was 303.9 ng/ml and the PIVKA-II level was 468 mAU/ml.

In January 2005, the AFP level was 93.7 ng/ml and the PIVKA-II level was 38 mAU/ml. The patient then experienced recurrent tumors in liver segment five, for which TACE was performed each time.

The patient remained in good health for about 6 years.

 

In July 2008, acute peritonitis developed due to tumor invasion into the duodenum and the patient died.

## 3. Discussion

Several treatments, such as TACE, PEIT, RFA, and hepatic resection, have been established as effective and safe therapeutic modalities for HCC [7,8]. However, frequent HCC recurrence, even after curative treatment, and advanced stages of HCC, such as in cases with portal invasion, remain major clinical problems.

The product UFT® combines tegafur, a prodrug of 5-fluorouracil, with uracil, a biomedical modulator, in a molar ratio of 4:1. UFT® has been reported to be effective against colorectal and lung adenocarcinomas as well as HCC. Therefore, the patient in this case report, with advanced multiple HCCs with portal vein tumor thrombi, was treated with UFT®. As a result, this patient showed marked tumor regression without side effects and had a good prognosis.

Recently, UFT® has received considerable attention as

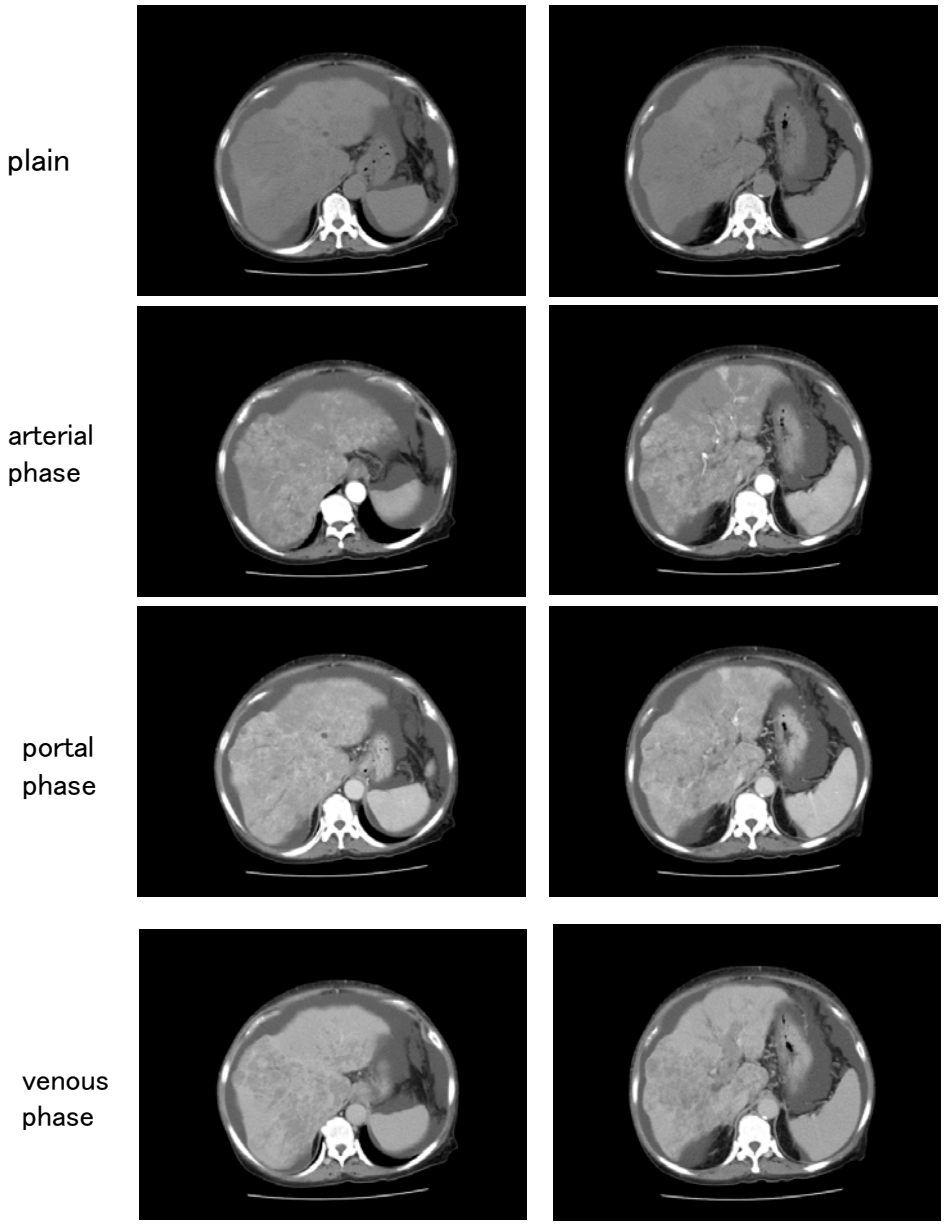


**Figure 1. A large mass occupies the entire liver and tumor thrombi are apparent in main portal vein. Moderate ascited is detected.**

 

**Table 1. Laboratory data on admission.**

| | | | |
|---|---|---|---|
| WBC | 8800/ul | BS | 14l mg/dl |
| Neutro | 60.9% | CRP | 2.52 mg/dl |
| Eosin | 3.6% | Na | 139 mEq/L |
| Baso | 0.5% | k | 3.6 mEq/L |
| Mono | 9.1% | CI | 99 mEq/L |
| Lympho | 25.9% | | |
| RBC | $508 \times 10^4$/ul | | |
| Hb | 16.1 g/dl | AFP | 317.2 ng/ml |
| Ht | 48.0% | PIVKA-II | 162210 Mau/ml |
| PIt | $15.6 \times 10^4$/ul | | |
| TP | 7.1 g/dl | | |
| Alb | 3.2 g/dl | | |
| BUN | 18 mg/dl | | |
| Cr | 0.7 mg/dl | | |
| T-Bil | 1.5 mg/dl | | |
| D-Bil | 0.6 mg/dl | | |
| AST | 122 IU/L | | |
| ALT | 39 IU/L | | |
| PT % | 53.3% | | |
| PT INR | | | |
| LDH | 357 U/L | | |
| γ -GTP | 330 U/L | | |

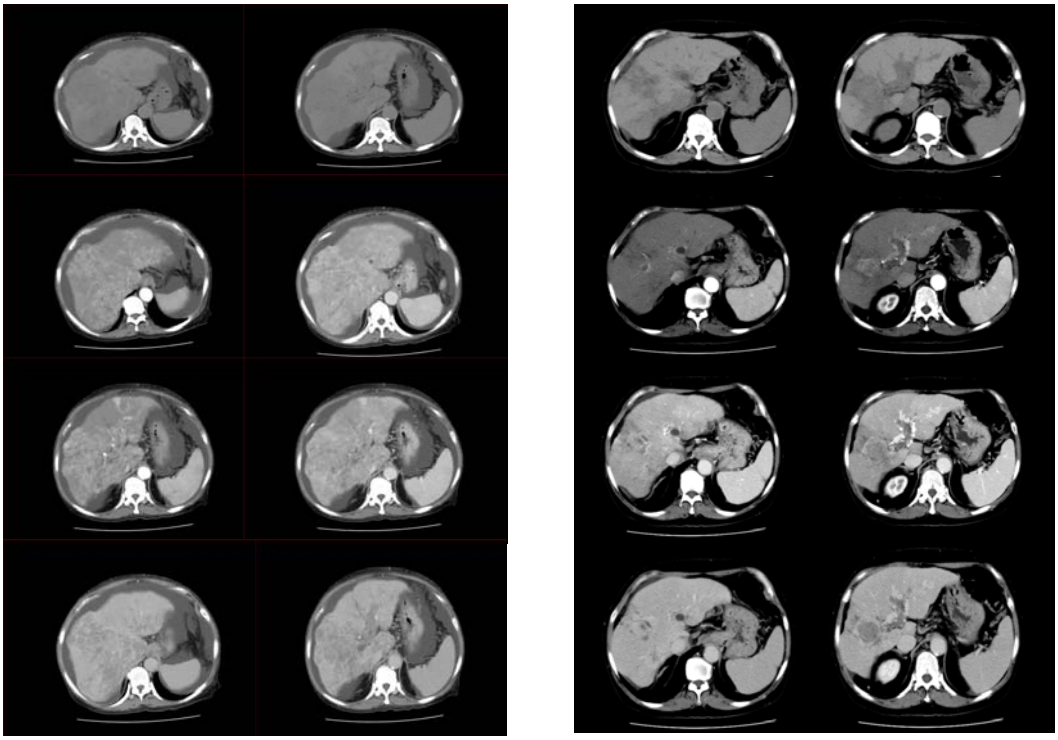

**Figure 2. A large mass occupied the entire liver become smaller 8 months after treatment. Though tumor thrombi in main portal vein do not disappear, cavernous transformation by portal vein is formed. Ascites is disappeared.**

 

an effective anticancer therapy [9-12]. However, the effectiveness of UFT® has been controversial, as some studies have suggested that the efficacy of UFT® may have been overestimated [13].

Tegafur is slowly metabolized by mitochondrial cytochrome 450 to 5-fluorouracil (5-FU) and the additional uracil potentiates the efficacy of tegafur by inhibiting its catabolism [14,15]. The mechanism of this inhibition by uracil is the blockage of dihydropyrimidine dehydrogenase (DPD) activity [16]. Therefore, rapid degradation of 5-FU by high levels of DPD activity in hepatoma cells has been implicated in 5-FU insensitivity. On the other hand, patients with low DPD activity in HCC tissues have a high concentration of 5-FU, which may have a potent anticancer effect against HCC. In this regard, Baba H *et al.* showed that some HCC tissues have low DPD activity and such HCC tissues may be 5-FU sensitive [17].

Recently, we showed that UFT® administration after TACE in cases of advanced HCC was advantageous and that it contributed to the inhibition of tumor angiogenesis through vascular endothelial growth factor (VEGF) [18]. In addition, some studies have shown that UFT® inhibits tumor angiogenesis in several cancer types. Our findings suggest that the HCC derived from the patient in this case report had low DPD activity and that tumor angiogenesis was effectively inhibited by UFT® treatment.

In conclusion, the present report demonstrates marked tumor regression and a good prognosis following oral UFT® treatment in a patient with advanced multiple HCCs with portal vein tumor thrombosis. This case strongly indicates that oral UFT® can be an effective treatment for some cases of advanced HCC with tumor thrombosis (VP4).

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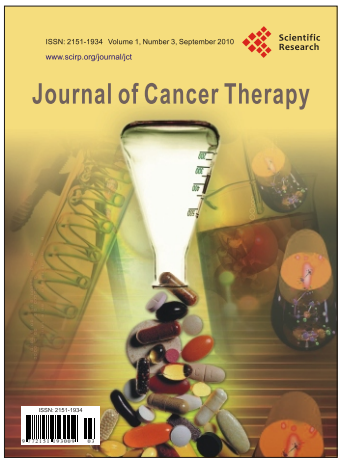


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