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**DRAFT**

**CC/05/10**

**COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD,  
CONSUMER PRODUCTS AND THE ENVIRONMENT.**

**CHOLANGIOCARCINOMA: PATHOGENESIS IN HUMANS AND RATS**

**Introduction**

1. Cholangiocarcinoma arises from the biliary epithelial cells of the common bile duct. The Oxford Textbook of Oncology states that cholangiocarcinoma classically refers to tumours of the epithelial cells of the intrahepatic bile ducts. Many researchers refer to cholangiocarcinomas according to position in the biliary tract such as intrahepatic, perihilar, distal extrahepatic (also referred to as bile duct carcinoma).<sup>2,3</sup> In humans, the most common form of biliary tract malignancy is adenocarcinoma of the gall bladder. Cholangiocarcinoma is usually highly malignant and is often diagnosed at an advanced stage where therapeutic measures have limited effect. Intrahepatic cholangiocarcinoma is also subdivided into morphological appearance as “mass-forming” and “infiltrating” subtypes. There is evidence for different clinical courses and gene expression profiles for these two types of cholangiocarcinoma. The COC reviewed the evidence for an increasing incidence of intrahepatic cholangiocarcinoma in February 2002 (Statement COC/02/S1). The conclusions reached are reported below:

*The Committee noted that a substantial increase in the reported rate of mortality from intrahepatic cholangiocarcinoma had been documented in both England and Wales and in the US over the last 30 years or so and concluded that changes in diagnostic standards over time could contribute to this increase. It was therefore important to undertake additional investigations before a definite conclusion could be reached about whether there had been real increase in the incidence of this tumour. The Committee recommended that:*

*i) an integrated pathological and clinical review of cases was needed to ascertain the accuracy of diagnosis of intrahepatic cholangiocarcinoma and, in particular, the potential for diagnostic transfer from secondary adenocarcinoma in the liver, for example, from carcinoma of the pancreas to intrahepatic cholangiocarcinoma.*

*ii) further evaluation of time-trends for intrahepatic cholangiocarcinoma in other countries, particularly with reference to the introduction of improvements in diagnostic imaging, would be helpful.*

*iii) consideration should be given to examination of information on the diagnosis of intrahepatic cholangiocarcinoma in a number of specialist liver units before and after the introduction of better diagnostic imaging techniques.*

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*iv) whether or not the recorded increase in incidence of intrahepatic cholangiocarcinoma was artefactual, it was clearly higher than was previously believed to be the case. Given the poor prognosis from this cancer, it was important to improve our understanding of its aetiology*

*v) the topic should be kept under review.*

2. The objective of this brief overview paper is to present a summary of current information on the pathogenesis of cholangiocarcinoma in humans and in rat models. The information is drawn from key reviews of the human data on cholangiocarcinoma, studies to investigate a rat model for cholangiocarcinoma and searches of the NTP/IARC databases. This information should provide background information for COC members in evaluating the human relevance of the finding of cholangiocarcinoma in carcinogenicity bioassays. The available information on human cholangiocarcinoma (etiology and pathogenesis) have been reviewed first. Further sections on cholangiocarcinoma in rats, including an overview of chemicals inducing cholangiocarcinoma derived from the NTP bioassay programme and the IARC monograph database, and an overview of information on pathogenesis in rats, have also been provided.

## **Cholangiocarcinoma in humans**

### Etiology

3. An overview has been recently published by Olnes and Erlich<sup>2</sup>. These authors subdivide risk factors according to weight of evidence. Holzinger et al (1999) grouped risk factors according to common predisposing factors.<sup>3</sup> The table below is an amalgamation of these two approaches.

### Strong association

Anatomic anomalies	Choledochal cyst, Caroli's disease Pancreobiliary maljunction (32x)* Congenital bile duct cysts (86x)
Chronic inflammatory	Primary Sclerosing Cholangitis (PSC) (30x) <i>Opisthorchis viverrini</i> , <i>Clonorchis sinensis</i> (5x)
Biliary calculi	Hepatoolithiasis Choleocystolithiasis
Radiological	Thorium dioxide, other radionucleotides (303x)
Autoimmune diseases	PSC (30x), Primary biliary cirrhosis (30x), chronic ulcerative cirrhosis (75x)

### Weak association

Carcinogens	Asbestos, PCBs, Nitrosamines (N-nitroso(2-oxopropyl)amine, DEN), betel nut, Isoniazid, Oral
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contraceptives, Cigarette smoking in patients with PSC.

\* estimated RR as cited by Holzinger et al 1999.

4. It is reported that biliary dysplasia may represent a precancerous lesion in patients with PSC. There is evidence for an increase aneuploidy in lesions arising in patients with PSC. The pathogenesis associated with infection with liver flukes is briefly included in the descriptions given below in order to allow members to compare this information with evidence on chemical induction of cholangiocarcinoma. The evidence on chemical exposure and induction of cholangiocarcinoma was considered by Olnes and Erlich to be weak and derived predominantly from case reports and carcinogenicity bioassays in rodents.<sup>2</sup> It was noted that there was epidemiological evidence that tobacco smoking was associated with progression of PSC to cholangiocarcinoma. Although evidence of an association with PCB exposure was considered, it was also reported that there was no evidence published to date for cholangiocarcinoma in the follow up studies of exposure to 2,3,7,8-TCDD and other dibenzo-p-dioxins/furans following the Seveso incident.<sup>2,3</sup>

5. Pathogenesis has been studied through histopathological examination of patients undergoing surgery and more recently through molecular approaches to gene expression changes in tissues obtained from patients. The Oxford text notes that although, histologically, cholangiocarcinoma is an adenocarcinoma arising from the intrahepatic biliary epithelium, it has abundant fibrous stroma. The majority of cholangiocarcinomas are tubular (77%) with 17% reported to be papillary. Mucin production is documented in many cholangiocarcinomas.<sup>1</sup>

6. Holzinger et al 1999 proposed four stages that are involved in the pathogenesis of cholangiocarcinoma<sup>3</sup>:

Stage 1: Predisposing factors, anatomic, chronic inflammation, infection with parasites, biliary calculi, exposure to carcinogens and autoimmune changes

Stage 2: Biochemical changes induced by chronic inflammation or cholestasis leading to DNA damage and mutations. Noted that *K-ras* mutations occur in 80-100% of patients with diagnosed cholangiocarcinoma. Allelic loss or point mutation in p53 reported in approximately 40% of patients with diagnosed cholangiocarcinoma.

Stage 3: Dysregulation of DNA repair and apoptosis leading to malignant transformation. Over expression of Bcl-2 reported in patients with cholangiocarcinoma.

Stage 4: Histological events. Evidence from a number of studies on carcinoma of the gallbladder and the papilla of Vater for a sequence of changes involving intestinal metaplasia leading to dysplasia and carcinoma. Other studies of patients undergoing cholecystectomies

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suggest an adenoma to carcinoma sequence. An evaluation of 58 patients with cancer of the ampulla of Vater showed epithelial dysplasia in all patients and adenoma structures in 91.4% of these patients. The authors noted that differentiation of bile duct tumours from those arising from the duodenal mucosa may be particularly difficult.

7. More recent reviews have summarised the available information on gene expression changes associated with cholangiocarcinoma in humans.<sup>2,4</sup> These are summarised below.

### ***Gene expression changes in human cholangiocarcinoma***

<b>Gene</b>	<b>Mechanism</b>
COX-2 COX-1	Overexpression, resistance to apoptosis, induction of EGFR, MAPK
K-ras	Overexpression
p53	Overexpression
c-erbB-2 (HER-2-neu)	Overexpression, gene amplification
c-Met	Overexpression
ets-1	Overexpression
HGF	Mitogenic effect, enhances metastasis
hMLH1	Microsatellite instability, LOH, methylation
Bcl-X, Mcl-1	Aberrant expression, resistance to apoptosis
IFN- $\gamma$ , Fas	Resistance to apoptosis
Emodin, caspase-3,-9	Resistance to apoptosis
APC	LOH, methylation
P16, INK4a-ARF locus	LOH, methylation, promoter methylation
TIMP-3	LOH, methylation
RASSF1A	LOH, methylation
Beta-catenin	Post-translational inhibition
TGF- $\beta$ 1, VEGF	Overexpression, Transcriptional activation
Smad4	Decreased expression
iNOS,NO	Induction of DNA damage

8. A lot of molecular pathology studies have been published in recent years. Although the data are not sufficiently robust to allow proposals to be made about the multi-stage process of cholangiocarcinogenesis, a number of authors have reported information from immunohistochemical investigations of both tumour and non-tumour tissue from patients with intrahepatic and extrahepatic carcinoma of the bile duct. They consider that this demonstrates some early gene-expression changes in tumourigenesis and some which may be associated with the later stages of tumour metastasis. [Informed consent was obtained in these studies]. It has been suggested that K-ras mutation is an early event whilst p53 mutation is a late event but the evidence is not conclusive.

9. There is some evidence from evaluation of immunohistochemistry of tissue obtained from 41 patients with extrahepatic bile duct carcinoma (obtained from the Department of Pathology, Osaka University Faculty of Medicine, Osaka, Japan) to suggest that over expression of ets-1 (which is involved in organ formation, tissue modelling, embryo development and tumour progression) is involved in the early malignant transformation and progression of bile duct carcinoma. 61% of patients with bile duct

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carcinoma were classified as ets-1 positive. Data from the same study of 19 patients with cholangiocarcinoma found that 21.6% were positive for ets-1. The expression of ets-1 showed an inverse relationship with Ki-67 labelling (a measure of tumour aggressiveness) and vascular and perineural invasion of bile duct carcinoma.<sup>5</sup> It was suggested that ets-1 expression was involved in the early phases of bile duct carcinoma.

10. An immunohistochemical investigation was carried out on tissue from patients (identified from stored tissues at the Department of Pathology, Kyushu University, Fukuoka, Japan).<sup>6</sup> The study included 23 cases with hepatolithiasis and 81 cases with intrahepatic cholangiocarcinoma (20 of whom also had metastatic lymph nodes). Positive staining for c-erbB-2 was noted in 61% of patients with hepatolithiasis and 55% of patients with cholangiocarcinoma. Positive staining for c-Met was reported in 35% of patients with hepatolithiasis and 38% of patients with cholangiocarcinoma. c-Met staining predominated in differentiated cholangiocarcinoma as compared to undifferentiated cholangiocarcinoma. c-Met staining was inversely correlated with tumour size, the presence of perineural invasion and presence of lymph node metastases. C-Met positive staining was associated with a longer survival. In contrast c-erbB-2 staining was reported in a high proportion of patients with lymph node metastasis (80%). The authors suggest that c-met expression is an early stage in cholangiocarcinogenesis whilst c-erbB-2 is associated with advanced stages of cholangiocarcinogenesis.

11. An immunohistochemistry evaluation was undertaken using archived paraffin blocks of liver specimens from 24 patients with cholangiocarcinoma and 7 apparently normal cases obtained from the Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.<sup>7</sup> A marked expression of COX-2 was identified in cholangiocarcinoma cells which was independent of the stage of tumour differentiation. COX-2 expression was also identified in normal hepatocytes. COX-1 expression in normal tissue was localised to Kupffer and endothelial cells and occasionally hepatocytes but was not seen in bile duct cells. A weak to moderate COX-1 expression was reported for cholangiocarcinoma and was also considerably enhanced in Kupffer cells of patients with cholangiocarcinoma.

12. The cause of cholangiocarcinoma in the cases reviewed above has not been reported. It is possible to derive some tentative conclusions on gene expression changes in early and later stages of cholangiocarcinoma in humans. One conclusion cited in one of the reviews considered in this paper was that chronic biliary inflammation and cholestasis, exposing the cholangiocyte to a milieu that provides an ongoing proliferative stimulus, results in reduced apoptosis and the accumulation of mutations. The pathogenesis was reported to involve the acquisition of autonomous growth signalling, loss of p53 survival pathway, overbalance towards antiapoptotic proteins, expression of telomerase, autonomous production of angiogenic factors and genetic changes associated with invasive phenotype (human aspartyl asparaginyl-hydroxylase (HAAH) expression).<sup>4</sup>

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**Cholangiocarcinoma in the rat**

13. The following chemicals which induce cholangiocarcinoma in rats were identified from searches of NTP or IARC databases and from PUBMED. This evidence shows that both non-genotoxic and genotoxic carcinogens may induce cholangiocarcinoma and often induce other liver tumours. In addition, cholangiocarcinoma has been induced in rats exposed to liver flukes.

***Chemicals inducing cholangiocarcinoma in rats***

<b>Chemical</b>	<b>Liver/Biliary tumours documented</b>
1-Aminodibromoanthraquinone (ref NTP TR 383, in-vitro mutagen)	Cholangiocarcinoma, hepatocellular adenoma and carcinoma in F344/N rats (both sexes)
Aflatoxin B1 (ref IARC, In-vivo mutagen)	Cholangiocarcinoma
Coumarin (Carlton BD et al 1996 (refs 8,9), ?mutagenicity)	Cholangiocarcinoma, other liver tumours noted.
DEN (Ha WS et al 2001, in-vivo mutagen) (ref 10)	Cholangiocarcinoma, hepatocellular carcinoma
Fuminosin B (IARC, in-vivo mutagen)	Cholangiocarcinoma in male rats
Furan (IARC, ? Mutagenicity)	Cholangiocarcinoma, hepatocellular adenoma and carcinoma in rats of both sexes.
Furfural (IARC, ? Mutagenicity)	Cholangiocarcinoma (rare occurrence in male rats), hepatocellular adenoma and carcinoma in rats.
o-nitrotoluene ( NTP TR 504? Mutagenicity)	Cholangiocarcinoma and hepatocholangiocarcinoma in male F344/N rats
PBBs (Firemaster) (IARC, not mutagenic)	Cholangiocarcinomas and other liver tumours in rats.
PCB 126 (3,3,4,4,5-PCB) and PCB 153 (2,2,4,4,5,5-PCB) (NTP 530, not mutagenic)	Cholangiocarcinoma, hepatocellular adenoma and carcinomas in female Harlan SPD rats.
PCB 126, PCB 118 (2,3,4,4,5-PCB) (NTP TR 531, not mutagenic)	Cholangiocarcinomas, hepatocellular adenomas, carcinomas.
2,3,7,8-TCDD (NTP TR 521, not mutagenic)	Cholangiocarcinomas, hepatocholangiocarcinoma, hepatoadenoma.
2,3,7,8-TCDD, 2,3,4,7,8-PCDF, 3,3,4,4,5-PCB (NTP 526, not mutagenic)	Cholangiocarcinoma, hepatoadenoma in female Harlan SPD rats.
Thioacetamide (IARC, in-vivo mutagen)	Cholangiocarcinoma and other liver tumours.
(Mutagenicity data surmised from	

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data retrieved from IARC/NTP summaries)	
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? mutagenicity = evidence for in-vitro mutagenicity, but in-vivo data either not reviewed or no published conclusion retrieved.

14. There is comparatively little information in the published literature on spontaneous cholangiocarcinoma in strains of rats used in carcinogenicity bioassays. A short section is presented in the NIEHS reference text on pathology of the Fisher rat.<sup>11</sup> The few spontaneous cholangiocarcinomas that have been observed in F344 rats consist of a moderately well-differentiated epithelium arranged in irregular branching ductular structures with scant stroma. In contrast, it is noted that chemically induced cholangiocarcinomas often contain abundant dense collagenous tissue. Histologically, cholangiocarcinomas are similar to cholangiofibrosis. Differentiation can be made on evidence of epithelial cell proliferation and invasive growth. In the F344 rat, cholangiocarcinomas are reported to be slow growing and of relatively low grade malignancy. Cholangiofibrosis has not been reported in control F344 rats. The term 'hepatocholangiocarcinoma' is used to refer to tumours which arise in the hepatic parenchyma and are considered subtypes of hepatocellular tumours. The term 'cholangioma' refers to a benign tumour of the bile duct, which presents with little evidence of fibrosis.

15. Two research papers have been retrieved which report the results of investigations into the pathogenesis of cholangiocarcinoma in Sprague-Dawley using thioacetamide<sup>12,13</sup> One of these studies also reported on a comparison of altered gene expression in mass-forming peripheral cholangiocarcinoma in patients undergoing resection and on cholangiocarcinoma from rats given thioacetamide in the drinking water.<sup>13</sup>

16. Briefly, male SPD rats were administered 300 mg/l thioacetamide in the drinking water for up to 24 weeks. A control group of 10 rats was used. Groups of 8 animals were sacrificed at weekly intervals from week 5 onwards. Blood samples were taken for AST, ALP, bilirubin and prothrombin measurements. The liver was perfused with paraformaldehyde. Liver sections were taken for H&E histology and immunohistochemistry for c-Met and c-erbB2 expression (and biliary cytokeratin CK19 expression, method not reported). Thioacetamide had no effect on AST, ALP, bilirubin or prothrombin measurements. Body weight was reduced, compared to controls, from week 8 onwards. Mortality was not increased by thioacetamide in this study. There was no evidence for any histological changes up to week 8. By week 9, ductular proliferation with demonstrable atypia (biliary dysplasia) was observed in 50% of rats. Abnormal luminal profiles with enlarged nuclei, hyperchromasia, pleomorphism, conspicuous nucleoli and loss of nuclear polarity were observed. Biliary dysplasia was accompanied in many instances by metaplastic intestinal-type goblet cells.

17. Progression of atypia without evidence of invasion was observed up to the 16<sup>th</sup> week at which time 50% of animals developed white, round and firm nodules on the surface of the liver (left, middle and right) lobes.

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Histopathology revealed intestinal-type cholangiocarcinoma with intense stromal desmoplasia (fibrosis). The neoplastic glands revealed all the features noted at week 9, including the presence of goblet cells. In addition, prominent intra-luminal necrosis was observed. Intense expression of biliary cytokeratin CK 19 was seen in all neoplastic glands. There was a heterogeneous distribution of lesions from week 16-22. During this period, the incidence of cholangiocarcinoma increased to 100% of all livers examined. Hepatocellular pathology was noted from week 20 and included hepatic fibrosis culminating in macronodular cirrhosis. No systemic metastasis was reported at week 24 when the study was terminated.

18. c-Met and c-erbB2 expression were increased in epithelial cells of early dysplastic glands (at week 9) as well as in later invasive cholangiocarcinomas. Expression was minimal or absent in normal ductular epithelium. The authors concluded that the pathology seen in this study was consistent with that documented in human cholangiocarcinoma.<sup>12</sup>

19. In a further experiment, the same research group administered 0.3% thioacetamide in drinking water to SPD rats for up to 26 weeks. Three experimental rats and one control rat were sacrificed every 2 weeks and immunohistochemistry of liver sections was undertaken. Microfoci of cancerous cells were observed from week 12 and gross evidence for tumours from week 16 onwards. All rats displayed diffuse mass forming cholangiocarcinoma after 24 weeks. There was no treatment related mortality during this study. Immunohistochemistry for EGFR, MUC1, MUC2 and MUC5AC, MMP-2, MMP-9 and p53 proteins was undertaken. Immunohistochemistry was also undertaken using sections prepared from 30 patients with mass-forming peripheral cholangiocarcinoma (26 were diagnosed as histologically adenocarcinoma, while the remaining 4 were adenosquamous carcinoma). The results reported by the authors are given below. The authors concluded that there was good agreement between gene expression in thioacetamide induced cholangiocarcinoma and in human cholangiocarcinoma.<sup>13</sup>

Tissue examined	EGFR	MUC1	MUC2	MUC5AC	MMP-2	MMP-9	P53
Human cholangiocarcinoma (30)	14 (47)	30 (100)	0	0	22(73)	22 (73)	9 (30)
Rat cholangiocarcinoma (n=24) (rats sacrificed from week 12 onwards)	24 (100)	24 (100)	0	0	24 (100)	24 (100)	0
Rat without cancer (n=15) (up to week 10)	2 (17)	0	0	2 (17)	0	0	0
Sham operation (n=13)	0	0	0	0	0	0	0

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20. There are some data relating to the pathogenesis of furan-induced cholangiocarcinoma in the published literature.<sup>14</sup> These have not been reviewed in detail in this paper. A key change in gene expression has been reported to involve over expression of growth factor tyrosine kinases c-erbB2/c-Neu and c-Met, together with aberrant autocrine expression of hepatocyte growth factor/scatter factor, the ligand for c-Met. It has been reported that the cyclo-oxygenase-2 inhibitor NS-398 produces dose dependent inhibition of rat cholangiocarcinoma cells *in-vitro*.

21. In separate experiments, an increase in serum levels of B10 alkaline phosphodiesterase (APDE) was reported to be a potentially useful early marker of cholangiocarcinoma in male F344 rats dosed with 3-methyl-4-dimethylaminoazobenzene and in Long-Evans Cinnamon rats (which spontaneously develop cholangiocarcinoma.)<sup>15,16</sup>

### Conclusion

22. The pathogenesis of human cholangiocarcinoma has been reviewed. A number of both genotoxic and non-genotoxic carcinogens can induce cholangiocarcinoma in rats. There is published evidence that the histological changes and gene expression changes in induced cholangiocarcinoma in rats (with some chemicals such as thioacetamide and furan) are similar to those reported in humans with cholangiocarcinoma, with evidence to suggest that dysplasia may be observed prior to the formation of carcinoma.

Secretariat May 2005

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