J Hepatobiliary Pancreat Surg (2006) 13:274–279
DOI 10.1007/s00534-005-1055-3

Journal of HBP Surgery

Role of epigenetic alterations in cholangiocarcinoma

Iris Tischoff, Christian Wittekind, and Andrea Tannapfel

Institute of Pathology, University of Leipzig, D-04103 Leipzig, Germany

Abstract
Intrahepatic cholangiocarcinomas are rare malignant epithelial liver tumors arising from intrahepatic bile ducts. The prognosis of affected patients is poor. Several risk factors, including hepatolithiasis, liver fluke infection, and anatomical abnormalities associated with inflammation of the biliary tract have been described. At present, little is known about the cellular and molecular mechanisms leading to the development of cholangiocarcinoma. In recent years, in addition to genetic alterations, epigenetic inactivation of (tumor suppressor) genes by promoter CpG island hypermethylation has been recognized as an important and alternative mechanism in tumorigenesis. This review discusses the epigenetic inactivation of different tumor suppressor genes in cholangiocarcinoma.

Key words Cholangiocarcinoma · Tumor suppressor genes · DNA methylation

Introduction

Cholangiocarcinoma (CC) is a rare primary malignant epithelial liver tumor arising from intrahepatic bile ducts. CC accounts for less than 2% of all malignancies and for about 5% to 10% of primary liver cancers.[1] Adenocarcinoma of the extrahepatic bile ducts (EBDs) is even less more common, by a factor of 2 to 5, compared to carcinomas of the gallbladder.

The incidence of CC is 2–4/100000 per year, and it has increased in recent years worldwide.[2] The highest incidence of CC is in Southeast and Eastern Asia. These areas have a high incidence of liver fluke infestation which increases the risk of CC by a factor of 25 to 50.[3–5] Hepatolithiasis, which is common in Asia, is also strongly associated with CC, with an incidence of approximately 5% to 10% of this tumor.[6] Anatomical abnormalities of the biliary tract, such as biliary atresia or Caroli's disease, leading to an inflammatory environment, are also associated with a higher incidence of CC.[7] Patients with predisposing risk factors develop CC as much as two decades earlier than those without such factors. Most CC, however, occurs in the absence of known etiological factors.

At present, little is known about molecular alterations in CC. As for many other tumors, the development of CC must also be understood as a multistep process, with the accumulation of genetic and epigenetic alterations in regulatory genes, leading to the activation of oncogenes and the inactivation or loss of tumor suppressor genes (TSGs). The genetic changes in CC identified so far include mutations of k-*ras*, *p53*, $p16^{INK4a}$, and *Smad4*; loss of heterozygosity (LOH) of *APC*; and allelic losses on 3p13-p21 and 8q22.[8–12]

In the past two decades, epigenetic inactivation of TSGs, through DNA methylation, has come into focus in studies of the development of malignant tumors, including CC.

DNA methylation

DNA methylation is a reversible chemical modification of the cytosine in CpG islands of promoter sequences, catalyzed by a family of DNA methyltransferases.[13,14] DNA methylation does not change the genetic information, it just alters the readability of the DNA and results in the inactivation of genes by subsequent transcript repression.

In humans and other mammals, CpG island methylation is an important physiological mechanism. The inactivated X-chromosome of females, silenced

Offprint requests to: C. Wittekind
Received: August 1, 2005 / Accepted: September 1, 2005

alleles of imprinted genes, and inserted viral genes and repeat elements are inactivated through promoter methylation.[15,16]

In human cancer, global hypomethylation of the genome exists in parallel to local hypermethylation in the promoter regions of TSGs.[17] Global hypomethylation in cancer causes genomic instability, and loss of imprinting, with biallelic expression, in, for example, *IGF2*. In contrast, promoter methylation is associated with the aberrant silencing of transcription, leading to the inactivation of TSGs or the biallelic suppression of imprinted genes, as has been described for *p57*.[18]

TSGs epigenetically silenced through DNA hypermethylation are involved in important molecular pathways of carcinogenesis, e.g., cell-cycle regulation, apoptosis, DNA repair, and cell adhesion.[19]

Proliferation and apoptosis

Among the genetic abnormalities that have been demonstrated in CC, *p53* mutations and activating k-*ras* mutations are the most frequent.[20] Alterations of the INK4a-ARF locus, located on 9p21, have been shown to contribute to the development of biliary tract tumors. This locus codes two cell-cycle regulatory proteins, $p16^{INK4ab}$ and $p14^{ARF}$, acting through the retinoblastoma (Rb)-cyclin-dependent protein kinase 4 (CDK4) and p53 pathways. $p16^{INK4a}$ binds to CDK4 and inhibits the ability of CDK4 to interact with cyclin D1. In the absence of $p16^{INK4a}$, CDK4 binds to cyclin D1 and phosphorylates *pRb*, leading to entry into the S phase.[21] This tumor suppressor gene (i.e., $p16^{INK4a}$) is frequently inactivated in a variety of tumors by deletion, mutations, and promoter hypermethylation. In CC, CpG island methylation appeared to be the main cause of $p16^{INK4a}$ inactivation. Methylation of $p16^{INK4a}$ was described in up to 83% of CCs.[22,23]

Our own observations demonstrated a strong correlation between $p16^{INK4a}$ methylation and k-*ras* mutations, suggesting a close molecular link between $p16^{INK4a}$ and k-*ras* in the tumorigenesis of CC. A similar association between *p53* mutations and promoter methylation of $p16^{INK4a}$ or $p14^{ARF}$ was not described.[24]

Further, $p16^{INK4a}$ hypermethylation seems to be an early and frequent event during neoplastic progression. Ishikawa et al.[25] demonstrated reduced $p16^{INK4a}$ protein expression due to $p16^{INK4a}$ promoter methylation in intraductal papillary neoplasm of the liver arising in hepatolithiasis.

The $p14^{ARF}$ gene induces cell-cycle arrest by preventing *p53* degradation through its binding to MDM-2. In our investigations, $p14^{ARF}$ hypermethylation occurred in 25% of CCs.[26] Moreover, inactivation of $p14^{ARF}$, irrespective of genetic or epigenetic events, seems to be a rare mechanism, suggesting that $p16^{NK4a}$ is the primary target gene of the INK4A/ARF locus in CCs.

Death-associated protein kinase (*DAPK*) is a proapoptotic gene that is involved in death receptor and mitochondrial pathways. Inactivation of *DAPK* decreases the induction of $p14^{ARF}$/*p53*, resulting in the inactivation of the *p53*-dependent apoptotic pathway.[26] *DAPK* is mostly inactivated through the hypermethylation of its promoter.[19] In CC, DNA methylation of *DAPK* is a rare event, and was described in about 8% of CCs.[23,27]

Cell adhesion

E-cadherin (*CDH1*) is a calcium-dependent cell-adhesion molecule that suppresses tumor-cell invasion and metastasis.[28,29] Downregulation of E-cadherin by genetic and epigenetic alterations has been reported in a variety of malignant tumors, including breast and gastric carcinomas. Moreover, re-expression in E-cadherin-negative cancer cell lines was induced by treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) suggesting that aberrant promoter methylation is one mechanism causing transcript suppression of E-cadherin.[30]

In CC, mutations of the E-cadherin gene are rare events, with a prevalence of 12%. Downregulation of E-cadherin is more commonly mediated through DNA methylation. Yang et al.[23] and Lee et al.[31] have described promoter methylation of E-cadherin in 21% and 48% of CCs, respectively.

DNA repair

Defects in DNA repair mechanisms may result in the accumulation of mutations and genomic instability.

The mismatch repair (MMR) system is one of the most important DNA repair mechanisms, correcting errors in DNA replication. Defects of the MMR system, leading to microsatellite instability (MSI), have been observed in approximately 15% of sporadic colorectal and gastric carcinomas.[32,33] Thorotrast-related CC showed a high MSI frequency, of up to 63%. The MSI phenotype showed a coincidence with *hMLH1* promoter methylation, suggesting that Thorotrast may induce MSI through hypermethylation of the *hMLH1* promoter.[34] Limpaiboon et al.[35] reported that in liver fluke-associated CC the *hMLH1* promoter was hypermethylated in 45% of the cases. Besides, 17% of the *hMLH1* methylated carcinomas showed LOH of *hMLH1*. A correlation of MSI and *hMLH1* methylation was also found.[35]

Abraham et al.[36] reported that MSI also occurred in biliary intraepithelial papillary neoplasms, but without *hMLH1* CpG island methylation.

The O6-methylguanine DNA methyltransferase (*MGMT*) is another important DNA repair gene protecting cells from DNA damage that is mediated by mutagenic and cytoxic agents, leading to alkylation at O6-guanine. Loss or reduced *MGMT* expression due to CpG island methylation was detected in several kinds of human cancers.[37,38] In CC, aberrant methylation was reported to occur at frequencies of 11% to 27%, respectively.[23,31]

The detoxificating glutathione S-transferase P1 (*GSTP1*) gene inactivates electrophilic carcinogens by conjugation with gluthatione. Promoter methylation of *GSTP1* is best analyzed in prostate cancer. Even in high-grade prostatic intraepithelial neoplasis, loss of *GSTP1* expression is caused by DNA methylation. Many other tumor types, including breast and hepatocellular carcinomas, showed a *GSTP1* hypermethylated promoter.[39–41] In CC, methylation of the *GSTP1* gene was a rare event, occurring in 6% and 34% of cases, respectively.[23,31]

Methylation hot spot chromosome 3p

Alterations of the genetic information on chromosome 3 are one of the most frequent and earliest steps in the carcinogenesis of several types of tumors, including primary malignant liver tumors. In hepatocellular carcinomas (HCCs), LOH of chromosome 3p occurred in about 30% of the patients, with 3p loss being significantly higher in CC than in HCC.[42,43] The short arm of human chromsome 3 is also one of the regional methylation hot spots, in addition to chromosomal locuses 11p and 17p.

Several genes on 3p, including *RASSF1A* at 3p21.3, *hMLH1* at 3p21.3, and the retinoic acid receptor β2 (*RARβ2*) gene at 3p24.2, are candidates for epigenetic inactivation through promoter hypermethylation.

RASSF1A is a bona fide multifunctional tumor suppressor gene that protects cells from genomic instability and transformation by stabilizing the microtubules.[44,45] Shivakumar et al.[46] have reported that *RASSF1A* blocks the cell cycle by inducing growth arrest with the inhibition of cyclin D1 protein accumulation. Recently, it has been shown that *RASSF1A* regulates the progression of mitosis by inhibiting the anaphase promoting complex activated by the regulatory subunit CDC 20 (APC-Cdc)20 complex and the stability of mitotic cyclins. Therefore, loss of *RASSF1A* may contribute to tumor progression by inducing both disturbance of mitotic progression and chromosome instability.[45,47] *RASSF1A* is one of the most common epigenetically inactivated genes. In about 50% of malignant tumors, this gene is methylated. The prevalence is highest in renal cell carcinoma, reaching about 91%.[48] In CC, hypermethylation occured in approxiamately 67% of cases.[31,43,49]

Two other putative tumor suppressor genes located on 3p21.3 are Semaphorin 3B (*SEMA3B*) and *BLU*.

Little is known about the *BLU* gene. In lung cancer, *BLU* overexpression inhibits tumor colony formation effiency. Qiu et al.[50] reported that *BLU* may function as an environmental stress-reponsive gene, regulated by E2F, at least in nasopharyngeal carcinomas. However, *BLU* methylation is rare in some types of epithelial tumors, e.g., in CC. We have recently found *BLU* promoter methylation in about 20% of our CC cases examined.[49]

SEMA3B is a member of the Semaphorin family, playing a role in axonal guidance and regeneration. It has been demonstrated that *SEMA3B* suppresses tumor formation in lung cancer and induces apoptosis. The latter is antagonized by $VEGF^{165}$, due to an interaction with neuropilin (NP)-1 receptor.[54–56] Recently, we reported a high prevalence of *SEMA36B* methylation in CC, reaching 100%. In contrast, non-neoplastic liver surrounding the tumor exhibited an unmethylated *SEMA3B* promoter.[49]

Our own data showed that *RASSF1A* and *SEMA3B* hypermethylation significantly correlated with LOH of *RASSF1A* and the *SEMA3B* locus at 3p21.3. Previous studies have demonstrated that mutations on 3p21.3 are infrequent events. It has also been shown that treatment with the demethylating drug 5-AZA-C restored *RASSF1A* and *SEMA3B* expression in cell lines, suggesting that promoter hypermethylation is responsible for silencing transcript expression.[44,49,54]

The (*RARβ2*) gene, located at 3p24.2, functions as a key retinoid receptor by mediating antiproliferative, differentiation, and apoptosis-inducing properties of retinoids. *RARβ2* expression is reduced in a large variety of malignant tumors. Methylation-induced silencing of this gene was described in lung and breast cancers.[57,58] In CC, promoter methylation of this gene occurred at a frequency of 16%.[23]

Cholangiocarcinoma (CC) versus adenocarcinoma of the extrahepatic bile ducts (EBDs)

Adenocarcinoma of the EBDs arises from the perihilar or distal EBDs. The annual incidence is below 1 in 100 000.[59] In contrast to CC, the etiological risk factors are uncommon. Predisposing conditions include bile duct stones, ulcerative colitis, and/or primary sclerosing cholangitis, as well as tobacco-associated conditions and choledochal cysts, to a lesser extent.[7,60]

The molecular pathogenesis of EBD adenocarcinoma is understood as less as the cancer-related alterations in

Table 1. Methylation in cholangiocarcinoma and extrahepatic bile duct carcinoma

Gene	Location	Function	CC	EBD carcinoma	Reference nos.
p16^{INK4a}	9q21	CDK inhibitor	17%–83%	43%–54%	22, 23, 31
p14^{ARF}	9q21	MDM2 inhibitor	25%–30%	46%	22, 23
p15^{INK4b}	9q21	CDK inhibitor	54%	48%	23
APC	5q21	β-Catenin inhibitor	27%–47%	44%	23, 31
p73	1p36.3	p53 Homologue	27%	43%	23
DAPK	9q34.1	Apoptosis	8%	6%–43%	23, 27, 31, 61
E-Cadherin	16q22.1	Cell adhesion	21%–48%	40%	23, 31
TIMP3	22q12	MMP inhibitor	9%	ND	23
hMLH1	3p21.3	Mismatch repair	18%–44%	32%	23, 34, 35
MGMT	10q26	DNA repair	11%–27%	33%–40%	23, 27, 31
GSTP1	11q13	Detoxification	34%	6%	23
THBS1	15q15	Anti-angiogenic	11%	ND	23
RASSF1A	3p21.3	Apoptosis	48%–67%	69%–83%	23, 43, 49, 62
BLU	3p21.3	Unknown	20%	ND	49
SEMA3B	3p21.3	Apoptosis	100%	ND	49
RARβ2	3p24.2	Retinoid effector	16%	20%	23

CC, cholangiocarcinoma; EBD, extrahepatic bile duct; ND, not done

CC. Comparing epigenetic alterations, the patterns of methylated genes in EBD adenocarcinoma are similar to those in CC. Yang et al.[23] analyzed the promoter methylation of 12 putative tumor-suppressor genes in 72 biliary duct cancers, consisting of equal numbers of CCs and EBD carcinomas. Promoter methylation of *DAPK*, *GSTP1*, and *RASSF1A* was more prevalent in adenocarcinoma of the EBDs than in CC (Table 1[23,61,62]).

In benign biliary epithelia specimens, included as controls, methylation of genes occurred only in individual cases. These results demonstrate that methylation is one of the major mechanisms for the inactivation of certain TSGs in CC and EBC adenocarcinoma. *p16^{INK4a}* promoter methylation occurred in about 43% and 54%, respectively,[23,63] of EBD carcinomas. In primary scleroscing cholangitis (PSC)-associated EBD carcinomas, *p16^{INK4a}* methylation was relatively rare. Mutations of the *p16^{INK4a}* promoter have been reported as the major inactivating mechanism in PSC.[64,65] The observation that *p16^{INK4a}* was epigenetically inactivated in 46% of patients with PSC without carcinoma[65] suggests that chronic inflammation leads to gains of methylation with a subsequent transcriptional inactivation of genes, e.g., those regulating the cell cycle. Similar findings were described in ulcerative colitis and chronic gastritis.[66,67]

Recently, Klump et al.[68] detected promoter methylation of *p16^{INK4a}* and *p14^{ARF}* in endoscopically obtained bile specimens from EBD, and concluded that the early and noninvasive detection of DNA methylation may serve as an indicator of the malignant potential of bile duct lesions, especially in patients with an increased risk for EBD cancer.

Aberrant DNA methylation of the repair gene *MGMT* was described in 33% to 40% of EBD carcinomas. Interestingly, transcriptional repression of *MGMT*, mediated by aberrant promoter methylation, was associated with the accumulation of GC-to-AT transitional mutations in the *p53* gene and — at a lower level — also in the k-*ras* gene.[23,27]

Summary

Emerging evidence exists, that gene silencing by CpG island methylation is a fundamental aspect of tumorigenesis. In accord with Knudson's two-hit gene-silencing hypothesis, it was clearly shown by several studies — including our own results — that sporadic tumors get losses of TSG by mutations in one allele, while the other allele is hypermethylated, leading to functional inactivation. Moreover, several TSGs may be inactivated by biallelic hypermethylation.

In CC, biallelic inactivation by hypermethylation, as well as allelic loss, has been shown for several TSGs, including *hMLH1*, *RASSF1A*, and *SEMA3B*.

Another aspect of DNA methylation is that epigenetic inactivation of DNA repair genes affects genetic alterations. MSI, mediated through aberrant promoter methylation of the *hMLH1* gene, was also described in CCs.

Moreover, epigenetic changes can occur at early neoplastic stages, and may serve as indicator lesions for the screening of patients with an increased risk for the disease.

References

1. Wittekind Ch. Hepatocellular carcinoma and cholangiocarcinoma. In: Hermanek P, Gospodarowicz MK, Henson DE, editors. Prognostic factors in cancer. Berlin Heidelberg New York Tokyo: Springer; 1995, p. 88–93.
2. Harder J, Blum HE. Cholangiocarcinoma. Schweiz Med Rundsch Prax 2002;91:1352–6.
3. Watanapa P. Cholangiocarcinoma in patients with opisthorchiasis. Br J Surg 1996;83:1062–4.
4. Shin HR, Lee CU, Park HJ, Seol SY, Chung JM, Choi HC, et al. Hepatitis B and C virus, *Clonorchensis sinensis* for risk of liver cancer: a case-control study in Pusan, Korea. Int J Epidemiol 1996;25:933–40.
5. Watanapa P, Watanapa WB. Liver fluke-associated cholangiocarcinoma. Br J Surg 2002;89:962–70.
6. Kubo S, Kinoshita H, Hirohashi K, Hamba H. Hepatolithiasis associated with cholangiocarcinoma. World J Surg 1995;19:637–41.
7. Lipsett PA, Pitt HA, Colombani PM, Boitnott JK, Cameron JL. Choledochal cyst disease. A changing pattern of presentation. Ann Surg 1994;220:644–52.
8. Kang YK, Kim WH, Lee HW, Lee HK, Kim Y. Mutation of *p53* and k-*ras* and loss of heterozygosity of *APC* in intrahepatic cholangiocarcinoma. Lab Invest 1999;79:477–83.
9. Yoshida S, Todoroki T, Ichikawa Y, Hanai S, Suzuki H, Hori M, et al. Mutations of *p16Ink4A/CDKN2* and *p15Ink4B/MTS2* genes in biliary tract cancers. Cancer Res 1995;55:2756–60.
10. Hahn SA, Bartsch D, Schroers A, Galehdari H, Becker M, Ramaswamy A, et al. Mutations of the *DPC4/Smad4* gene in biliary tract carcinoma. Cancer Res 1998;58:1124–6.
11. Shiraishi K, Okita K, Kusano N, Harada T, Kondoh S, Okita S, et al. A comparison of DNA copy number changes detected by comparative genomic hybridization in malignancies of the liver, biliary tract and pancreas. Oncology 2001;60:151–61.
12. Kawaki J, Miyazaki M, Iro H, Nakagawa K, Shimizu H, Yoshidome H, et al. Allelic loss in human intrahepatic cholangiocarcinoma: correlation between chromosome 8p22 and tumor progression. Int J Cancer 2000;88:228–31.
13. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetic joins genetic. Trends Genet 2000;16:168–74.
14. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247–57.
15. Riggs AD, Pfeifer GP. X-chromosome in activation and cell memory. Trends Genet 1992;8:169–74.
16. Razin A, Cedar H. DNA methylation and genomic imprinting. Cell 1994;77:473–6.
17. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983;301:89–92.
18. Feinberg AP. Genomic imprinting and cancer. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. pp. 525–37.
19. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61: 3225–9.
20. Tannapfel A, Weinans L, Geissler F, Schütz A, Katalinic A, Kockerling F, et al. Mutations of *p53* tumor suppressor gene, apoptosis and proliferation in intrahepatic cholangiocarcinoma of the liver. Dig Dis Sci 2000;45:317–24.
21. Liggett WH Jr, Sidransky D. Role of the *P16* tumor suppressor gene in cancer. J Clin Oncol 1998;16:1197–206.
22. Tannapfel A, Sommerer F, Benicke M, Weinans L, Katalinic A, GeiMßler F, et al. Genetic and epigenetic alterations of the INK4a-ARF pathway in cholangiocarcinoma. J Pathol 2002;197: 624–31.
23. Yang B, House MG, Mingzhou G, Herman JG, Clark DP. Promoter methylation profiles of tumor suppressor genes in intrahepatic and extrahepatic cholangiocarcinoma. Mod Pathol 2005; 18:412–20.
24. Tannapfel A, Benicke M, Katalinic A, Uhlmann D, Köckerling F, Hauss J, et al. Frequency of $p16^{INK4A}$ alterations and k-*ras* mutations in intrahepatic cholangiocarcinoma of the liver. Gut 2000;47:721–7.
25. Ishikawa A, Sasaki M, Sato Y, Ohira Sh, Chen MF, Huang SF, et al. Frequent $p16^{ink4a}$ inactivation is an early and frequent event of intraductal papillary neoplasm of the liver arising in hepatolithiasis. Hum Pathol 2004;35:1505–14.
26. Raveh T, Kimchi A. DAP kinase — a proapoptotic gene that functions as a tumor suppressor. Exp Cell Res 2001;264:185–92.
27. Koga Y, Kitajima Y, Miyoshi A, Sato K, Kitahara K, Soejima H, et al. Tumor progression through epigenetic gene silencing of O^6-methylguanine-DNA methyltransferase in human biliary tract cancers. Ann Surg Oncol 2005;12:1–10.
28. Sommers CL, Thompson EW, Torri JA, Kemler R, Gelmann EP, Byers SW. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. Cell Growth Differ 1991;2:365–72.
29. Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayashi T, et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissue and its relationship to metastasis. Cancer Res 1993;53:1696–701.
30. Endo K, Ashida K, Miyake N, Terada T. E-cadherin gene mutations in human intrahepatic cholangiocarcinoma. J Pathol 2001;193:310–17.
31. Lee S, Kim WH, Jung HY, Yang MH, Kang GH. Aberrant CpG island methylation of multiple genes in intrahepatic cholangiocarcinoma. Am J Pathol 2002;161:1015–22.
32. Kim H, Jen J, Vogelstein B, Hamilton SR. Clinical and pathological characteristics of sporadic carcinomas with microsatellite sequences. Am J Pathol 1994;145:148–56.
33. Oliveria C, Seruca R, Sexias M, Sobrinho-Simoes M. The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different genes; a study of the *TGFβ*, *IGF2R*, and the *BAX* genes. Am J Pathol 1998; 153:1211–19.
34. Liu D, Momoi H, Li L, Ishikawa Y, Fukumoto M. Microsatellite instability in Thorotrast-induced human intrahepatic cholangiocarcinoma. Int J Cancer 2002;102:366–71.
35. Limpaiboon T, Khaenam P, Chinnasri P, Soonklang M, Jearanaikoon P, Sripa B, et al. Promoter hypermethylation is a major event of *hMLH1* gene inactivation in liver fluke related cholangiocarcinoma. Cancer Lett 2005;217:213–9.
36. Abraham SC, Lee JH, Boitnott JK, Agani P, Furth EE, Wu TT. Microsatellite instability in intraductal papillary neoplasmas of the biliary tract. Mod Pathol 2002;12:1309–17.
37. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 2000;60:829–5.
38. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-mehthylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasma. Cancer Res 1999;59:793–7.
39. Brooks JD, Weinstein M, Lin X, Sun Y, Pin SS, Bova GS. CG island methylation changes near the *GSTP1* gene in prostatic intraepithelial neoplasia. Cancer Epidemiol Biomarkers Prev 1998;7:531–6.
40. Esteller M, Corn PG, Urena JP, Gabrielson E, Baylin SB, Herman JG. Inactivation of gluthatione-S-transferase P1 gene promoter hypermethylation in human neoplasia. Cancer Res 1998;58:4515–18.
41. Tchou JC, Lin X, Freije D, Isaacs WB, Brooks JD, Rashid A, et al. GSTP-1 CpG island DNA hypermethylation in hepatocellular carcinomas. Int J Oncol 2000;16:663–76.

42. Shiraishi K, Okita K, Kusano N, Harada T, Kondoh S, Okita S, et al. A comparison of DNA copy number changes detected by comparative genomic hybridization in malignancies of the liver, biliary tract and pancreas. Oncology 2001;60:151–61.
43. Wong N, Li L, Tsang K, Lai PBS, To KF, Johnson PJ. Frequent loss of chromosome 3p and hypermethylation of *RASSF1A* in cholangiocarcinoma. J Hepatol 2002;37:633–9.
44. Damman R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a Ras association domain family protein from the lung tumor suppressor locus 3p21.3. Nat Genet 2000;25:315–9.
45. Liu L, Tommasi S, Lee DH, Dammann R, Pfeifer GP. Control of microtubule stability by the RASSF1A tumor suppressor. Oncogene 2003;22:8125–36.
46. Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA. The RASSF1A tumor suppressor blocks cell cylce progression and inhibits cyclin D1 accumulation. Mol Cell Biol 2002;22:4309–18.
47. Song MS, Song SJ, Ayad NG, Chang JS, Lee JH, Homg HK, et al. The tumour suppressor *RASSF1A* regulates mitosis by inhibiting the APC-Cdc20 complex. Nat Cell Biol 2004;6:129–37.
48. Dreijerink K, Braga E, Kuzmin I, Geil L, Duh FM, Angeloni D, et al. The candidate tumor suppressor gene, *RASSF1A*, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci USA 2001;98:3563–7.
49. Tischoff I, Markwarth A, Witzigmann H, Uhlmann D, Hauss J, Mirmohammadsadegh A, et al. Allele loss and epigenetic inactivation of 3p21.3 in malignant liver tumors. Int J Cancer 2005;115:684–9.
50. Qui GH, Tan LK, Loh KS, Lim CY, Srivastava G, Tsai ST, et al. The candidate tumor suppressor gene *BLU*, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress reponsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. Oncogene 2004;23:4793–806.
51. Agathanggelou A, Dallol A, Zöchbauer-Müller S, Morrissey C, Honorio S, Hesson L, et al. Epigenetic inactivation of the candidate 3p21.3 suppressor gene *BLU* in human cancers. Oncogene 2003;22:1580–8.
52. Hesson L, Bièche I, Krex D, Criniere E, Hoang-Xuan K, Maher ER, et al. Frequent epigenetic inactivation of *RASSF1A* and *BLU* genes located within the critical 3p21.3 region in gliomas. Oncogene 2004;23:2408–19.
53. Liu XQ, Chen HK, Zhang XS, Pan ZG, Li A, Feng QS, et al. Alterations of *BLU*, a candidate tumor suppressor gene on chromosome 3p21.3, in human nasopharyngeal carcinoma. Int J Cancer 2003;106:60–5.
54. Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Williams NN, et al. Allelic loss on chromosome 3p21.3 and promoter hypermethylation of Semaphorin 3B in non-small cell lung cancer. Cancer Res 2003;63:3352–5.
55. Tomizawa Y, Sekido Y, Kondo M, Gao B, Yokota J, Roche J, et al. Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene *SEMA3B*. Proc Natl Acad Sci USA 2001;98:13 954–9.
56. Castro-Rivera E, Ran S, Thorpe P, Minna JD.Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas $VEGF_{165}$ antagonizes this effect. Proc Natl Acad Sci USA 2004;101:11 432–7.
57. Virmani AK, Rathi A, Zöchbauer-Müller S, Sacchi N, Fukuyama Y, Bryant D, et al. Promoter methylation and silencing of the retinoic acid receptor-beta in lung cancer. J Natl Cancer Inst 2000;16:1303–7.
58. Widschwendter M, Berger J, Hermann M, Müller H, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-b2 gene in breast cancer. J Natl Cancer Inst 2000;10:826–32.
59. Liu C, Wang J, Ou QJ. Possible stem cell origin of human cholangiocarcinoma. World J Gastroenterol 2002;2:10–19.
60. Rosen CB, Nagorney DM, Wiesner RH, Coffey RJ Jr, LaRusso NF. Cholangiocarcinoma complicating primary sclerosing cholangitis. Ann Surg 1991;213:21–5.
61. Tozawa T, Tamura G, Honda T, Nawata SH, Kimura W, Makino N, et al. Promoter hypermethylation of DAP-kinase is associated with poor survival of primary biliary tract carcinoma patients. Cancer Sci 2004;95:736–40.
62. Chen YJ, Tang QB, Zou SQ Inactivation of *RASSF1A*, the tumor suppressor gene at 3p21.3 in extrahepatic cholangiocarcinoma. World J Gastroenterol 2005;11:1333–8.
63. Caca K, Feisthammel J, Klee K, Tannapfel A, Witzigmann H, Wittekind CH, et al. Inactivation of the INK4A/ARF locus and p53 in sporadic extrahepatic bile duct cancers and bile tract cancer cell lines. Int J Cancer 2002;97:481–8.
64. Ahrendt SA, Eisenberger CF, Yip L, Rashid A, Chow JT, Pitt HA, et al. Chromosome 9p21 loss and p16 inactivation in primary sclerosing cholangitis-associated cholangiocarcinoma. J Surg Res 1999;84:88–93.
65. Taniai M, Higuchi H, Burgart LJ, Gores GJ. $p16^{INK4a}$ promoter mutations are frequent in primary sclerosing cholangitis (PSC) and PSC-associated cholangiocarcinoma. J Gastroenterol 2002;123:1090–8.
66. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-ralated CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573–7.
67. Jang TJ, Kim DI, Shin YM, Chang HK, Yang CH. *p16* (*INK4a*) promoter hypermethylation of non-tumorous tissue adjacent to gastric cancer is correlated with glandular atrophy and chronic inflammation. Int J Cancer 2001;93:629–34.
68. Klump B, Hsieh CJ, Dette S, Holzmann KH, Sinn U, Ortner M, et al. Promoter methylation of INK4a/ARF as detected in bile — significance for the differential diagnosis in biliary disease. Clin Cancer Res 2003;1773–8.