Biochemical Indicators of Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is one of the most frequent epithelial liver tumors and a significant problem with regard to clinical management. The causes of CCA are heterogeneous and associated with the geographic factors, with particularly high incidence in East Asian countries. Patients with CCA often suffer from adverse clinical outcomes and a poor prognosis, much of

which is due to the lack of efficient diagnostic tools and biomarkers. Moreover, genome-wide screening of gene alterations and omics analyses demonstrate the highly diverse nature of oncogenic genetic factors among individual CCA tumor cells. Here, we summarize the current approaches to identifying CCA tumor biomarkers and other biochemical indicators for diagnosis and selection of appropriate treatment as a step toward precision medicine with personalized patient care and a goal of improved clinical outcomes.

Keywords

CA19-9
CEA
Cell-free DNA
CTC
Microvesicles
Mucins
Noncoding RNA
MicroRNA
Gene therapy

Abbreviations

AFP	Alpha-fetoprotein
AID	Activation-induced cytidine deaminase
AMPN	Aminopeptidase N
APC	Adenomatous polyposis coli
APOBEC	Apolipoprotein B mRNA editing enzyme
ARID1A	AT-rich interactive domain-containing protein 1A
AUC	Area under the curve
BAP1	BRCA1-associated protein 1
BAR	Beta-catenin/armadillo-related protein
B-CADHERIN	XXX

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BCL2	B-cell lymphoma 2
BCLX5	B-cell lymphoma-extra large 5
BCLXL	B-cell lymphoma-extra large
BRAF	v-Raf murine sarcoma viral oncogene homolog AQ3
BRCA1	Breast cancer susceptibility gene I
BRCA2	Breast cancer susceptibility gene II
BSEP	Bile salt export pump
CA 19-9	Carbohydrate antigen 19-9
CCA	Cholangiocarcinoma
CCND1	Cyclin D1
CDC6	Cell division cycle 6
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2
CEA	Carcinoembryonic antigen
cfDNA	Cell-free DNA
COX-2	Cyclooxygenase 2
CTC	Circulating tumor cell
CYP1A2	Cytochrome P450 family 1 subfamily A member 2
dCCA	Distal cholangiocarcinoma
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ERB-2	Erb-B2 receptor tyrosine kinase 2
EVs	Extracellular vesicles
FBXW7	F-box and WD repeat domain containing 7
FGF19	Fibroblast growth factor 19
FGFR2	Fibroblast growth factor receptor 2
FIC1	Familial intrahepatic cholestasis type 1
FXR	Farnesoid X receptor
GANP	Germinal center-associated nuclear protein AQ4

GSTO1	Glutathione S-transferase omega 1
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HOXD9	Homeobox protein Hox-D9
iCCA	Intrahepatic cholangiocarcinoma
IDH1	Isocitrate dehydrogenase [ADP(+) 1]
IDH2	Isocitrate dehydrogenase [ADP(+) 2]
KEAP1	Kelch-like ECH-associated protein 1
K-RAS	Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LTO1	LTO1 maturation factor of ABCE1
mAb	Monoclonal antibody
MCL1	Induced myeloid leukemia cell differentiation protein
MDM2	Mouse double minute 2 homolog
MDR3	Multidrug resistance gene 3
miR	MicroRNA
MRP2	Multidrug resistance-associated protein 2
MTHFR	Methylenetetrahydrofolate reductase
MVs	Microvesicles
MYC	v-Myc myelocytomatosis viral oncogene homolog
NAT2	Arylamine N-acetyltransferase 2
ncRNA	Noncoding RNA
NF1	Neurofibromin 1
NKG2D	Natural killer cell lectin-like receptor subfamily 2D
OPCML	Opioid binding protein/cell adhesion molecule-like
p14arf	An alternative reading frame product of the CDKN2A locus
p16	Cyclin-dependent kinase inhibitor protein p16 (INK4a)
PBRM1	Protein polybromo-1
pCCA	Perihilar cholangiocarcinoma
PD-1/PD-L2	Programmed cell death 1/programmed death ligand 2
PI3KCA	Phosphoinositide 3-kinase p110

PIGR	Polymeric immunoglobulin receptor
PTEN	Phosphatase and tensin homolog
RAD51AP1	RAD51 associating protein-1
RASSF1A	Ras association domain family 1 isoform A
ROS1	ROS proto-oncogene 1
shRNA	Short hairpin RNA
SMAD4	Small body mothers against decapentaplegic 4 AQ5
SOCS3	Suppressor of cytokine signaling 3
SXIN-1	Stress-activated map kinase-interacting protein 1 AQ6
TGF-β	Transforming growth factor-β
TP53	Tumor protein p53
TYMS	Thymidylate synthetase
UNG	Uracil nucleotide glycosidase
VNN1	Vanin1
XRCC1	X-ray repair cross-complementing protein 1

Introduction

Cholangiocarcinoma (CCA) is the second most common liver malignancy worldwide and is particularly common in Southeast Asian countries such as Thailand, Cambodia, and Laos. A major feature of CCA is its heterogeneity, including of its risk factors and causes. For example, many cases of CCA in Northeastern Thailand are related to liver fluke infection and presumably originate over time as a result of inflammatory damage by the stenosis in the bile duct(s) [1, 2, 3]. In contrast, CCAs in the other countries are accompanied with sporadic genetic abnormalities commonly detected in various malignancies (oncogenic genetic alteration) [4, 5]. This heterogeneity of CCA complicates many aspects of its clinical management, including diagnosis, prognosis, and surveillance.

Early biomarker analysis of patient sera led to the finding that carbohydrate antigen 19-9 (CA19-9) and other carcinoembryonic markers are valid for the diagnosis and follow-up of CCA patients [6]. Genome-wide next-generation

gene sequencing (NGS) of CCA tumor cells has facilitated the accumulation of further information and formulation of a more comprehensive classification of CCAs as compared to earlier compendia of genetic data. Here, we show currently available standard biomarkers in sections "Classification of CCA", "Classical Serum Markers", and "Earlier Analyses of Gene Mutation and Amplification in CCA" and propose the advanced biomarker/gene marker strategy by combination with the data of the genome alterations of the individual CCAs in sections "Challenges of Highly Specific Biomarkers", and "Recent Classification of CCAs with Large-Scale Analysis of Multi-omics".
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Classification of CCA

The clinical diagnosis of CCA involves tumor classification depending on tumor location: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA). (i) Primary sclerosing cholangitis (PSC) is one of the primary causes of CCA development in the western world. It is a chronic disease of the intrahepatic and extrahepatic bile ducts due to inflammation and scarring. (ii) Parasitic infestation with *Opisthorchis viverrini* and *Clonorchis sinensis* is the significant risk factors of CCA in Asia, particularly in Southeast Asia. Due to the food-consumption behavior of humans to eat raw or undercooked fish, worms can infect humans via ingestion and inhabit in the bile ducts, gallbladder, and pancreatic duct [7]. (iii) Hepatolithiasis is one type of the gallstone disease with the stones in the intrahepatic bile ducts proximal to either the left or right hepatic duct. Hepatolithiasis-associated CCA with a high incidence in East Asian countries, such as Taiwan, China, Hong Kong, South Korea, and Japan [8, 9, 10, 11], may arise after the prolonged inflammation (recurrent or chronic inflammatory) of bile duct epithelium [12, 13]. (iv) Hepatitis virus infections, especially with hepatitis B and C viruses (HBV and HCV), are the causes of hepatocellular carcinoma (HCC). The epidemiologic evidence suggests chronic HBV and HCV infection may be involved in an increased incidence of iCCA [14, 15, 16].
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Classical Serum Markers

CCA is a "silent killer" in part due to the difficulty of being diagnosed before the advanced or metastatic stage. The CCA diagnosis depends on various

components, including clinical findings, imaging techniques, biochemical data, and histological information. Standard serum liver tests occasionally find initial changes in CCA (e.g., ALP, ALT, and total bilirubin). The current serum biomarkers for CCA are CA 19-9, carcinoembryonic antigen (CEA), mucins, and alpha-fetoprotein (AFP) and are reviewed herein (Table 6.1).

Table 6.1

The diagnostic value of serum markers for CCA

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Marker	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accu (%
CA 19-9	129 U/mL	79.0	98.0	78.6	99.4	96.2
	125.1 U/mL	76.7	80.0	79.3	77.4	–
	>37 KU/L	77.1	84.8	65.9	90.7	82.7
CEA	12.1 μg/L	53.3	86.7	80.0	65.0	70.0
	22 μg/L	68.6	81.5	58.5	87.2	78.0
CA 19-9 + CEA	–	76.7	83.3	81.1	78.1	80.0
CA 19-9 + AFP	–	86.7	83.3	83.9	86.2	85.0
CEA + AFP	–	83.0	87.0	86.2	83.9	85.0
MUC4	–	27.0	93.0	82.0	51.0	–
cfDNA						
OPCML	–	80.0	90.0	88.9	81.9	85.0
HOXD9	–	67.5	90.0	87.1	73.5	78.8
OPCML + HOXD9	–	62.5	100.0	100.0	71.7	81.3
MicroRNA						

Bile miR-191 + miR-486-3p + miR-1274b + miR-16 + miR-484	–	67.0	96.0	94.4	74.4	–

PPV positive predictive value, *NPV* negative predictive value

Carbohydrate antigen 19-9 (CA 19-9) is an epitope on the sialyl-Lewis antigens (Lew^a and Lew^b, etc.) which are produced by biliary, pancreatic, gastric, colonic, endometrial, and salivary epithelial cells [17]. It is a routine diagnostic marker for hepatobiliary and pancreatic malignancies, while it is not applicable for the tumors in the Lew^{a-} Lew^{b-} patients [18]. CA19-9 is a CCA marker with relatively high sensitivity (approx 79%) and specificity (approx 98%) when using a cutoff value of 129 U/mL [19]. Other cutoff values have been studied (e.g., in the context of PSC), and performance characteristics may depend on the presence of other underlying disease(s). Indeed, CA19-9 level may also increase in patients with cholangitis or pancreatobiliary ductal obstruction; therefore, CCA prediction by serum CA 19-9 is only reliable if combined with and interpreted in the context of other clinical data [20].

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Carcinoembryonic antigen (CEA) is a cell membrane-associated glycoprotein and shows the different expression patterns between healthy tissues and cancer cells. While it is a marker for colorectal and other adenocarcinomas [21, 22], CEA indicates approximately 53% sensitivity and 87% specificity for CCA diagnosis [23]. If combined with CA19-9, CEA gives valuable information on CCA prediction with 63% sensitivity and 87% specificity [4]. Besides, CEA is useful to predict the long-term survival after resection of CCA [24], and the higher expression levels of CEA and CA19-9 are related to the reduced overall survival of CCA patients [23].

Mucins are the glycoproteins secreted into the extracellular space from the epithelial cells. Various human malignancies show the changes in the expression levels of transmembrane mucins of MUC1, MUC2, MUC4, MUC5AC, MUC13, and MUC16 [25]. MUC4 expression increased highly and significantly in the advanced CCA patients of the poor prognosis group. MUC4 has a sensitivity (approx 27%) and specificity (approx 93%) for CCA [26]. Another biomarker,

MUC5AC, is expressed in the bronchial, gastric, and endocervix epithelium, but not in the normal intrahepatic biliary tree. Serum MUC5AC is positive in CCA patients with a 2.5-fold higher risk of death [27]. The ratio of MUC5AC expression between serum and bile is useful for the differential diagnosis of CCAs from cholangitis and biliary stones [28].

Serum alpha-fetoprotein (AFP) is a useful marker for hepatocellular carcinoma (HCC) and can be also for other cancers (gastrointestinal, pancreatic, biliary, nonseminomatous germ cell testicular, and germ cell ovarian cancers) [29]. Approximately 20% of CCA patients show a high AFP level (>20 ng/mL) [30]. AFP shows a high specificity for HCC diagnosis but low specificity (and sensitivity) for CCA diagnosis. However, AFP can be useful in combination with either one of the other markers such as CA19-9 (86.67% sensitivity and 83.33% specificity), CA125 (80.00% sensitivity and 86.67% specificity), CEA (83.33% sensitivity and 86.67% specificity), and CA242 (88.90% sensitivity and 89.7% specificity) [31, 32].

Earlier Analyses of Gene Mutation and Amplification in CCA

Analysis of gene expression and SNP microarray of CCAs demonstrated various alterations in oncogenic signaling pathways of CCND1 and FGF19 genes (amplification), KRAS and BRAF genes (mutation), and activation of inflammatory signaling pathways. The genetic alterations also classified CCAs into congenital abnormality group and acquired mutation group (Table 6.2) [33]. The current biomarker study may not be directly applicable as a diagnostic tool to predict the prognosis and the future severity of the disease, but may be useful when used in combination with the genetic study such as the number of affected genes and the critically impaired genes in CCAs. The five reports of clinical cases calculated higher-frequency gene alterations [34, 35, 36, 37, 38]; these included molecules of tumor suppressors (SMAD4, TP53, RASSF1A), transcription regulation (ARID1A), cell growth (BAP1, CDKN2A), oncogene [ERB-2 (HER2), K-RAS, B-RAF], protease protein degradation (FBXW7), glucose metabolism (IDH1, IDH2), and signal transduction (PBRM1, PI3KCA). A study of circulating tumor cell (CTC) DNA confirmed the high degree correlation of mutational frequencies with published datasets of CCA in TP53 (38-8%), ARID1A (36-4%), KRAS (28-5%), IDH1 (32-4%), BAP1 (29-1%),

PBRM1 (21-1%), SMAD4 (9-4%), PIK3CA (9-3%), and CDKN2A (7-0%) [39].

Table 6.2

Molecular abnormalities associated with CCA

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Origin/normal function	Gene (and/or protein)
I. *Congenital abnormality*	
Transport	MDR3, BSEP, MRP2, FIC1
Metabolism	CYP1A2, GST01, ARY2, BAR (FXR)
DNA repair and modification	MTHFR, TYMS, XRCC1
Tumor surveillance	NKG2D, COX-2
II. *Acquired mutation*	
Tumor suppressor	APC, BRCA1, E-CADHERIN, p16, p14arf, PTEN, RASSSF1A, **SMAD4**[a], **TP53**[a]
Transcription regulation	**ARID1A**[a], KEAP1
Apoptosis	AXIN1, BCL2, BCLXL, BCLX5, MCL1
Cell growth	**BAP1**[a], CCND1, CDK6, FGFR2, ROS1, **CDKN2A**[a]
Oncogene	BRAF, B-CADHERIN, EGFR (ERB1), **ERB2**[a] (HER2), **K-RAS**[a], MDM2, MYC
DNA damage and repair	BRCA2, RAD51AP1
Regulation of bile production	FGF19
Proteasomal protein degradation	**FBXW7**[a]
Glucose metabolism	**IDH1**[a], **IDH2**[a]
Ribosome biosynthesis	LTO1
Signal transduction	NF1, **PBRM1**[a], **PI3KCA**, SOCS3
Ref: Peter et al. [65]	

[a]Boldface indicates genes of high mutation frequency

Previous studies with smaller numbers of CCA cases attempted to identify the critical differences in gene alterations to account for the geographic differences in CCA development and prognosis. As mentioned earlier, CCAs are highly heterogeneous, in large part due to the etiological, environmental, anatomical, cellular, and genetic factors. CCAs with liver fluke infection, for example, are different than those of non-liver fluke-infected patients. Therefore, it has been difficult to discover the most appropriate biomarker among the conventional ones (or a novel one). Researchers have probably studied CCAs as different category tumors originating with different genetic alterations; though this may have offered some advantages, it may have also somewhat impeded understanding and generalization of genetic changes and clinical treatment of CCA.

Challenges of Highly Specific Biomarkers

Many technologies have been applied to improve diagnostic accuracy, particularly at an early stage of disease, and in order to best target therapeutic options. These are reviewed in the forthcoming paragraphs. Extracellular vesicles (EVs) are classified into microvesicles (MVs) and exosomes according to their size and biogenesis. MVs directly bud from the plasma membrane, and their size ranges between 100 and 1000 nm. Exosomes originate from multivesicular bodies; their size is smaller than 100 nm, and they float at a density of 1.13–1.19 g ml^{-1} in sucrose gradients [40, 41, 42]. EVs are found in blood, urine, saliva, bile, and ascites. They carry the disease biomarkers as specific proteins, lipids, RNA species, DNA, and metabolite [42, 43]. Proteomics analysis evaluated the abundance of oncogenic proteins in CCA cell-derived EVs and found aminopeptidase N (AMPN), vanin1 (VNN1), and polymeric immunoglobulin receptor (PIGR) in early-stage CCA to have an area under the curve (AUC) of 0.88, 0.88, and 0.84, respectively. The miR profiles from extracellular vesicles from human bile revealed miR-based panels of miR-191, miR-486-3p, miR-1274b, miR-16, and miR-484 to be valuable for CCA diagnosis, with 67% sensitivity and 96% specificity, which gives similar diagnostic potential compared to CA19-9 with the specified cutoff value [44].

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Circulating nucleic acid as cell-free DNA (cfDNA) is released in plasma and other body fluids from tumor cells. Investigation of cfDNA can assess the genetic and epigenetic alterations of individual patients, particularly those who are diagnosed in advanced stages of the disease and with limited therapeutic options. It can determine single-nucleotide mutations [45, 46, 47, 48], aberrations in DNA methylation [49, 50], copy number aberrations [45, 50, 51], and gene expression alterations [52, 53]. For example, molecular analysis of cfDNA can help screen for FGFR2 mutations both in the primary tumor and metastatic lesions to examine the gain of drug resistance against the BFJ398 inhibitor in patients with ICC. Multiple mutations of the FGFR2 gene affect the FGFR2 kinase domain and create a significant oncogenic alteration in cancer cells that should be critically monitored for decision-making in clinical treatment [54]. Other epigenetic changes, including DNA methylation, also provide useful information, such as cfDNA hypermethylation of opioid binding protein/cell adhesion molecule-like (OPCML: AUC, 0.85; sensitivity, 80%; specificity, 90%; accuracy, 85%) and homeobox protein Hox-D9 (HOXD9: AUC, 0.789; sensitivity, 67.5%; specificity, 90%; accuracy, 78.75%), to differentially diagnose CCA with a higher percentage compared to other biliary diseases [55].

Circulating noncoding RNA (ncRNA) may regulate gene transcription, transcript stability, and translation of protein-coding transcripts [56]. Altered expressions of serum and plasma miR-21 and miR-26a in patients are useful for diagnosis and the prediction of patient survival [57, 58]. CCA patient serum also shows the downregulation of miR-150-5p expression [59, 60]. MicroRNA is aberrantly expressed in CCAs as well as in all types of human tumors [61].

Circulating tumor cells (CTCs) overexpress epithelial cell adhesion molecule (EpCAM), an indicator of having lost cell-to-cell adhesion capacity and a propensity for metastasis. CCA has high-level expression of EpCAM, with a sensitivity of 93.7%, as shown by enrichment-immunofluorescence in situ hybridization (SE-iFISH) [62].

Recent Classification of CCAs with Large-Scale Analysis of Multi-omics

Application of NGS and omics examination for larger-scale clinical samples has

provided a comprehensive concept in the classification of CCAs. A world cooperative study of the combined datasets of a large whole-genome sequencing, whole-exome sequencing, copy number alterations, transcriptomes, and epigenomes proposed the classification of CCAs of 489 cases from 10 countries. It classified four types of CCA clusters based on tumor etiologies, anatomical locations, and the cellular origin, the tumor characteristics, and clinical features, as summarized below [63].

The first factor for clustering is history of liver fluke infection, and the second factor is genomic modification caused by increased DNA hypermethylation of CpG island shores and high levels of mutations in H3K27me3-associated promoters. Cluster 1, liver fluke (+)/genomic modification (-), and Cluster 2, liver fluke (+)/genomic modification (+), show recurrent mutations of TP53, ARID1A and BRCA1/2, and ERBB2 amplification. These clusters show a poor prognosis. Cluster 3, liver fluke (-)/genomic modification (-), and Cluster 4, liver fluke (-)/genomic modification (+), show recurrent mutations in epigenetic-related genes, i.e., BAP1 and IDH1/2, as well as FGFR rearrangements, and have high PD-1/PD-L2 expression. Clusters 3 and 4 show better prognosis. The higher-rate alterations of various genes in CCAs during clinical treatment are one of the causes of the worse prognosis.

Development of CCA is associated with metabolic syndrome, hepatolithiasis, congenital biliary tract malformations, bile duct cysts with the risk factors of chronic inflammation involving the biliary tract, several toxic and environmental factors such as nitrosamine-contaminated food, asbestos, dioxins, vinyl chlorides and thorotrast, smoking, and alcohol intake [32, 64]. Exposure to various long-term risk factors results in the proliferation and genetic and epigenetic alterations of cholangiocytes and their malignant transformation [64, 65].

Additionally, any stimulus that causes oxidative stress can be oncogenic. Several CCA cell lines induce aberrant expression of activation-induced cytidine deaminase (AID) as an initiator of gene alteration [66]. AID initiates somatic hypermutation at the immunoglobulin V-region gene and class switch regions in B lymphocytes [67]. The cytidine deamination potentially causes the DNA injuries, resulting in mutation of the promoter regions and coding regions of various critical genes by the assist of the associated protein complex of RNA

polymerase II, transcription elongation factor Spt5, UNG, and GANP [68, 69].

Current studies suggested that aberrant expression of cytidine deaminase molecules resulted in altering the genome. TGF-β stimulation causes the aberrant AID expression in multiple cancer cells of the digestive system [70, 71, 72]. Virus infection may evoke the APOBEC family cytidine deaminase proteins as the endogenous defense molecules [68]. Typically, human APOBEC3B is involved in the development of breast cancers [73]. These cytidine deaminase molecules enter into the nucleus together with the RNA exportation component GANP [69]. Aberrant expression of the APOBEC family cytidine deaminase protein associated with GANP might alter the genome randomly at the transcription-competent nucleosome, resulting in chromosome translocation, gene deletion, and mutations [72, 73, 74]. Therefore, it is difficult to predict and determine the genome alteration in individual CCA cells. Nevertheless, identification of novel biomarkers is an essential issue to monitor the gene mutations and chromosome modifications.

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Future Perspective

Combination of Standard Cancer Biomarker Plus CCA-Selective Biomarkers

Diagnosis and treatment of patients with CCA needs to be guided and advanced by incorporation of comprehensive tools, including with conventional biomarkers as well as patient-specific genetic and cellular abnormalities. As an initial tool, CA19-9 can be useful but still provides only a small piece of the overall picture and future clinical course. Preoperative or postoperative genetic information of tumor cells provides a vital avenue for better treatment. Surgical specimens provide the most information on genetic abnormalities of primary CCA foci. One approach to optimize nonsurgical specimens is to enrich CTCs in peripheral blood or ascites fluid by the antibody trap method using high-affinity monoclonal antibodies against a standard biomarker that appears commonly in CCA tumors (e.g., CA19-9, Muc5a). Antibody trapping of CTCs from CCA patients with magnetic beads enhances the tumor cells that are actively mutating and metastasizing in the cancer patient. Direct capturing of tumor cells under the microscope also provides insight regarding genetic alterations of the

individual primary CCA lesion. Such tumor cells demonstrate the genetic alterations and the critical causes of a highly malignant trait of cell proliferation, drug resistance, mutation, and anti-apoptotic properties. The genetic information includes the alterations in tumor suppressor genes such as p53, PTEN, p16, and CDC6 and the methylation status of CpG islands at the promoter regions of selected target genes; changes for CTNNB1, WNT5B, and AKT gene expression; somatic gene copy numbers of immune cells; mutations of BAP1 and IDH1/2; and the upregulation of FGFRs and PI3K signaling. AQ14

Cancer Therapy with an Infusion of Tumor Suppressor Genes and shRNA Vectors

Gene transfection therapy is one of the promising procedures for targeting of specific tumors. To develop next-generation cancer treatment, we need to solve two kinds of complicated issues. First, authentic and precise targeting depends on the identification of abnormalities of individual cancer cells. This is a cost-consuming and probably the most challenging issue for advanced clinical treatment. Practically, the procedure needs to target the most common genetic alterations associated with oncogenesis and cell proliferation. Gene alterations of CCAs include congenital molecular abnormalities of various functions such as molecular transport, cell metabolism, DNA repair and modification, and tumor surveillance as well as acquired mutations of multiple genes. The target genes for this procedure are diverse; therefore, rapid and convenient screening of individual patients is necessary. At present, however, we need to select several active target genes for the practical use.

The second issue is vector selection for gene therapy. The choices for vectors are many and will develop more in the future. One candidate vector is the lentivirus vector, which is highly effective in introducing genes into tumor cells [75]. The gene knockdown procedure into cancer cells is capable of targeting a cancer-specific abnormality with short hairpin RNA. Also, the human telomerase gene promoter can facilitate targeting of cancer cells [76]. One of the challenges of vector selection is as follows: the regular protocol of gene therapy is with drip infusion of five genes to the cancer patients in The Gene Osaka Clinic Inc. (http://www.g-cg.jp/) (Osaka, Japan). Drip infusion of five constructs (ten million virus titers/each gene at one time in a week) is

undertaken five or six times as a single course under the informed consent. The patient receives the course at least twice. The clinical outcome of CCA patients often shows marked decrease of cancer biomarkers with p53, PTEN, p16, CDC6-shRNA, and Gankirin-shRNA in combination with standard cancer therapy [77]. In principle, gene therapy causes the adverse side effect of virus particle infusion-associated inflammation and possibly allergic response; this includes fever (~38 °C), nausea, diarrhea, vomiting, and hypotension. In some cases, transient increases of ALT, AST, CRP, and white blood cell count occur. AQ15

The promise held by gene therapy against CCA will growingly contribute to the improvement of patient outcomes. The exchange of information, knowledge, and technical skills are necessary for this and other advancements in CCA treatment.

Conclusion

A hopeful approach for better cancer treatment depends on rapid and accurate diagnosis and targeted therapy based on individual cancer genetic information. Strategic biomarkers play an important role in this regard and are a subject of continued research.

Acknowledgments

The authors thank Drs. Sopit Wongkham and Atit Silsirivanit (Khon Kaen University, Thailand) for continuous support and encouragement.

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