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SPECIAL ARTICLE

Identification of Tumor Markers for Cholangiocarcinoma and Evaluation of Their Diagnostic Potential

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SUMMARY **Results obtained from studies using experimental animal model clearly showed that (1) A marker(s) for CCA does exist; 2) This marker is a glycoprotein with a molecular weight of 200 kDa; (3) It is produced and secreted *in vitro* by tumor cell lines; (4) It is highly immunogenic in mice and the MAb specific for this antigen is directed against the carbohydrate moiety; (5) This tumor antigen can be detected in serum and bile of tumor-bearing animals by a sandwich ELISA employing this MAb; (6) Kinetic studies show a gradual elevation of this antigen during tumor development; and (7) The elevation of this antigen can be detected at a time when no pathological changes have yet taken place, as judged by microscopic examination. Preliminary work from the human counterpart using human cholangiocarcinoma cell line showed promising results. CCA-specific antigen could be similarly identified and the MAbs produced were highly specific for this 160 kDa antigen.**

The incidence of liver cancer in Southeast Asian countries is among the highest in the world, with a total of more than 30,000 new cases per year. Of the two major types of primary liver cancer, that of the biliary system, ie, cholangiocarcinoma, occurs at higher frequency in areas where liver fluke infections are endemic. These infections in humans are caused by *Opisthorchis viverrini, O.felineus* and *Clonorchis sinensis.*[1] In Korea, a hepatocellular-carcinoma (HCC) to cholangiocarcinoma (CCA) ratio of 4:1 was found in the Pusan area where *C.sinensis* infection occurs, while a ratio of 10:1 was found in Seoul where *C. sinensis* does not occur.[2] Similarly, in Northeastern Thailand where endemic *O.viverrini* infection may be as high as 90% in some villages, a HCC: CCA ratio of between 3:1 to 4:1 has been reported. This compares with a ratio of 5:1 or 10:1 throughout the country.[3]

Different lines of evidence convincingly show a strong causal relationship between CCA and *O. viverrini* infection in humans.[1] In addition to the epidemiological data mentioned above, a number of retrospective and prospective studies conducted by different groups of investigators support such a causal association.[4-6] This has been supported by a number of studies using an experimental animal model, eg, Syrian golden hamsters infected with human liver flukes. In this animal model, pioneered by a group of investigators in Thailand,[7] a tumor having characteristics compatible with the CCA occurring in humans develops in infected animals that receive a subcarcinogenic dose of dimethylnitrosamine (DMN) in drinking water. Nitrosamine is a carcinogen found in the diet consumed by individuals residing in several tropical countries where CCA is commonly found. It should be mentioned at this point that nitrosamines need not come from exogenous sources only. They can also be synthesized endogenously from various precursors. Possible mechanisms for the development of CCA in infected hosts have been reviewed

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recently.[1] In brief, these possibilities include induction of a localized chronic inflammatory reaction, biliary proliferation and hyperplasia, alteration of the metabolism of carcinogens and stimulation of a host immune response which can make the host more susceptible to cancer induction and progression.

Cholangiocarcinoma remains an important public health problem in Thailand because the fatality rate is still very high, largely due to lack of early diagnosis and treatment. Patients with CCA are often diagnosed clinically and cholangiographically, but mostly when it is too late for the tumor to be managed successfully. Currently there is no specific and practical laboratory method for the diagnosis of CCA at its earliest stages, although work is underway to develop one. For example, many groups of investigators have reported that levels of several common tumor markers (including carcinoembryonic antigen [CEA], and a number of other different carbohydrate antigens) are significantly elevated.[8] However, these markers are not specific for CCA and the elevated levels are often detected only in cases where the tumor is either very large or has already metastasized. A combined measurement using more than one carbohydrate marker can increase the percentage of successful diagnoses, but this percentage is still far from being satisfactory.[9] Therefore, without a biopsy, these methods are only presumptive and, even with the biopsy, it is reliable only when the tumor mass is large enough for detection.

A definitive diagnosis for the early detection of CCA is urgently needed. It is for this reason that our groups at Mahidol University and Chulabhorn Research Institute in Thailand have made a considerable effort to develop a sensitive and specific definitive method for detecting CCA at its very early stages of development. The purpose of this communication is to describe the overall objective of our research plan and to summarize the results that we have achieved during the last 3–4 years.

Although the ultimate goal of our collaborative research effort is to develop a definitive laboratory method that will be highly reliable in the diagnosis of CCA at its early stages of development in man, the main discussion in this communication relates to our findings obtained with the animal model described above. This is because it is considerably more difficult to collect appropriate clinical specimens (eg, tumor tissues) from surgical patients. It would also be extremely difficult, if not impossible with human subjects, to perform kinetic studies during the carcinogenic process. We hope that the information obtained from the animal model will provide the insights needed to assess the potential of specific markers for early diagnosis of the tumor in humans and to provide guidelines for more systematic investigations to achieve that goal.

Identification and characterization of potential tumor markers in a hamster model

1. Induction of cholangiocarcinoma in hamsters: CCA was induced in Syrian golden hamsters, aged 3–4 weeks, essentially as originally described by Thamavit and his associates[7] and as outlined in the accompanying flow chart (Fig. 1). With this protocol, the tumor developed in a large majority of animals within 3–4 months after the termination of DMN treatment. The intrahepatic tumors were surgically removed and small portions were sent for microscopic examination to ascertain that they were cholangiocarcinoma. The remainders were used for other purposes, as outlined in the diagram. The tumor was also propagated to serve as an additional source of antigen by allotransplanting small liver tumor fragments, by either the subcutaneous or intramuscular route, into the hindleg of young adult hamsters. Within 2 – 3 months, new tumors developed to the size of 1 – 2 cm in 40 – 50% of the recipients. The allotransplanted tumors (AT) exhibited histopathological features similar to those of the original tumors (OT) that developed following liver fluke infection and DMN treatment. The AT could be surgically removed and similarly retransplanted into new recipients. It has been noted that the AT seemed to develop faster following retransplantation. Using this protocol, we have been able to keep the tumors propagated in hamsters until now, for a period of over one year.

2. Establishment of permanent cell lines from OT and AT tissues: Tumor tissues were removed, minced into fine fragments and seeded into 25 cm^2 plastic tissue culture flasks containing Ham's F12 culture medium supplemented with 10% foetal bovine serum, 10 ng/ml epithelial growth factor and a mixture of insulin, transferrin and selenium (manuscript submitted for publication). Within 4 weeks, aggregated tumor cell colonies could be observed. Contaminating fibroblasts were removed by differential trypsinization as described previously.[10] Two hamster tumor cell lines (one from OT and another from AT) have been maintained for over 80 passages during 3 years of *in vitro* culture. The population doubling times were not noticeably different for the two lines (ie, 31 hours for OT and 26 hours for AT). Both cell lines exhibited morphological features of epithelial cells, ie, they were uniformly polygonal and had a cobblestone-like appearance. Ultrastructural studies with scanning and transmission electron microscopes showed the presence of microvilli covered with glycoproteins or mucopolysaccharides, and microvillus-lined intracytoplasmic lumens containing secreted pro-

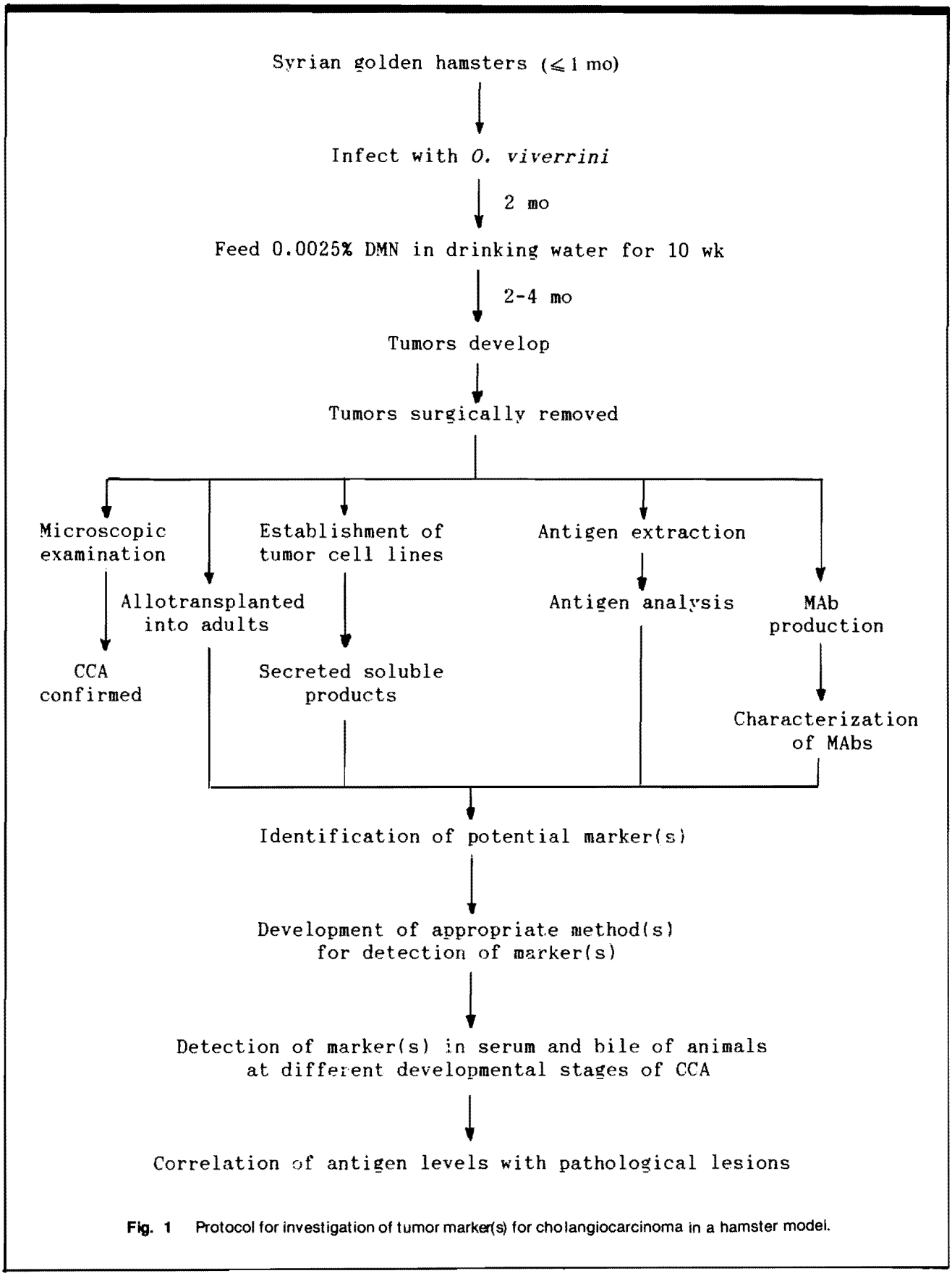

Fig. 1 Protocol for investigation of tumor marker(s) for cholangiocarcinoma in a hamster model.

ducts. Cytoplasmic microfilaments typical for cytokeratin fibers found in other epithelial cells could be readily visualized.

3. Production of rabbit polyclonal antibodies and mouse monoclonal antibodies (MAbs) to tumor antigens : The polyclonal antibodies used in our studies (eg, for antigen analysis and sandwich ELISA for the quantitation of tumor antigens) were produced by immunizing rabbits with tumor cell suspensions as described elsewhere.[11] For some experiments, the IgG fraction was prepared by ammonium sulfate precipitation, followed by ion-exchange chromatography on a DEAE column. A portion of the purified IgG fraction was conjugated with biotin and used in a sandwich ELISA.[12]

Monoclonal antibodies against the tumor antigens were produced by immunizing BALB/c mice subcutaneously with crude aqueous extracts of the AT homogenate.[12] The antibodies specific for CCA antigens were screened by indirect ELISA against crude tumor and normal tissue antigens. High titre antibodies that reacted specifically with tumor antigens were identified and characterized further by immunoblotting and immunohistochemical staining.

4. Extraction, purification and characterization of tumor antigens : Aqueous crude extracts were prepared from OT and AT by homogenizing the tumor fragments in small volumes of 5 mM EDTA and the supernatant fluids obtained after centrifugation were kept in small aliquots at $-20°C$ until used. A similar procedure was used for the preparation of normal tissue extracts serving as negative controls. A few recipients of the OT tumor developed mucin-filled cystadenomas instead of solid tumors. The mucinous fluids collected from these lesions were used as additional sources of antigen. We noted earlier and will describe in detail in other sections, that a glycoprotein component with a molecular weight of around 200 kDa was one CCA-associated antigen that was present in a relatively large quantity in the mucinous fluid. Therefore, the fluid was a rich source of 200-kDa antigen that could readily be purified. Following clarification by centrifugation at high speed, the clear mucinous fluid was applied to a Sephadex G-200 column. Fractions eluted in the void volume were collected and found to contain an immunoreactive 200 kDa glycoprotein in a relatively pure state. These 200 kDa enriched fractions were pooled and they have been used as a reference throughout our studies, eg, for construction of a standard curve required during the quantitation of antigen in specimens from tumor-bearing animals.

Antigens from OT and AT were similarly extracted and characterized. Crude homogenates and secreted products from these two cell lines were prepared by culturing the monolayers in serum-free culture medium for 2 days and collecting the spent culture media. The latter was exhaustively dialyzed to remove residual amino acids and other nutrients and then concentrated by lyophilization.

Crude tumor tissue and cell line homogenates and their secreted products were analyzed by different physicochemical and immunological methods in order to identify a component(s) that might be specifically associated with CCA. These methods included SDS-PAGE, immunoblotting, immunohistological stainings, lectin bindings, etc.

5. Identification and characterization of CCA-associated antigen(s) : Our initial attempts by classical physicochemical approach to identify components in the tumor tissue that were not present in normal tissues did not work because the system was too complicated. The possible presence of a few unique tumor components, most likely in trace quantities, was masked by normal tissue components present in much larger quantities in both tissues. Therefore, we next attempted to use immunochemical methods, using different polyclonal rabbit antisera. Again, this approach did not give satisfactory results because the antisera were dominated by antibodies to serum proteins and normal tissue antigens. Thus, in order to simplify the work even further, we attempted to produce monoclonal antibodies (MAbs) against the tumor antigens. From several MAbs reactive with these antigens, we picked the one which reacted most strongly with the tumor antigen in the indirect ELISA developed for our study.[12]

This MAb, produced by clone 6E5, was subsequently shown to react specifically with an extract component that migrated to the 200 kDa position in SDS-PAGE and in immunoblots. This immunoreactive component also reacted with wheat germ agglutinin, indicating that it was a glycoprotein. More detailed analysis using several other lectins suggested that it contained *N*-acetylglucosamine and/or sialic acid. The MAb 6E5 appeared to react with the carbohydrate moiety of the glycoprotein as evidenced by the disappearance of the reaction when the carbohydrate was oxidized by sodium-*m*-periodate under conditions known to selectively affect only the carbohydrate moiety. An attempt was also made to remove the carbohydrate side chain(s) from the core protein by treating the 200 kDa enriched fraction with trifluoromethane sulfonic acid and preliminary results were consistent with our expectations (ie, they indicated that the reactive epitope of the 200 kDa tumor antigen was located in the carbohydrate side chain).

Immunohistochemical studies employing both immunoperoxidase and immunofluorescent staining on

both the tumor sections and cell line monolayers were used in order to localize the 200 kDa tumor antigen. Results from these studies showed a strong reaction at the apical border of the tumor cells.[12] A weaker reaction was noted inside the cytoplasm and in the surrounding extracellular matrix. The MAb also reacted strongly with materials found in the lumen of proliferating bile ducts inside the tumor tissue, a finding consistent with data showing that the antigen was secreted in large quantity in the bile of tumor-bearing animals (to be discussed in the following section). This tumor antigen was also secreted by the two tumor cell lines, judging from immunoblotting and ELISA reactivity of their spent culture medium with MAb 6E5.

6. Evaluating the potential of the 200 kDa antigen for early detection of experimentally induced cholangiocarcinoma in hamsters: We felt that the data obtained from this experimental model would give valuable information that would never be possible from the study of CCA in humans. With the animal model, we could possibly follow the appearance and gradual elevation of this tumor marker from the very beginning of carcinoma induction, ie, immediately after exposure to the carcinogen. Moreover, groups of animals could be sacrificed and the livers examined for early pathological changes. It would therefore be possible to correlate the levels of the 200 kDa antigen with the stages of tumor development in each individual animal. Such studies could be executed in a limited time frame. If one were to attempt this in humans, a very long term prospective study on a very large group of high-risk subjects would be required. Even then, not as much useful information would be obtained. For instance, it would not be possible to do liver biopsies for correlation studies.

The MAb 6E5 was used for development of a detection method to quantitate the 200 kDa antigen because it reacted most strongly with the antigen in immunoblots.[12] The method developed was a MAb-based ELISA, modified from our original ELISA previously described for the quantitation of *O. viverrini* antigen.[13] The method was described in detail very recently by our group.[12] It could detect the antigen at concentrations as low as 10 ng/ml.

Next, we had to choose appropriate specimens to determine whether our method was sensitive enough to detect any possible elevation that might be occurring in tumor-bearing animals. Serum specimens was our first choice because they are easily obtained and would be the most useful and practical should the method ever be applied for routine diagnosis in humans. However, we also kept bile in mind because this is a tumor of the bile duct epithelium and one would logically expect to find the antigen secreted in the bile. If it was secreted in sufficient quantity into the bile, the detection of antigen in fecal specimens might even be possible.

Data presented in Table 1 represent the mean values obtained from serum and bile specimens accumulated from animals used in some of our experiments. It is clear that the antigen was present in serum and bile from tumor-bearing animals at concentrations that could be readily measured by our method. The results obtained showed that there was a marked elevation of the antigen following infection and DMN treatment. In fact, for paired specimens from the same animals, the antigen was found to be present in the bile at much higher concentration than in the serum. Fig. 1 shows a significant correlation between the antigen levels in these two body fluids. When the results from histopathological examination of animals in tumor induction experiments were considered, the data showed that the 200 kDa antigen levels were much higher in the induction group of animals with tumors than in controls. At the same time, even the non-tumor animals in the tumor induction group had significantly

Table 1. Correlation between pathological lesions and levels of 200 kDa antigen*

Experimental groups	Serum (ng/ml)		Bile (μg/ml)	
	No. specimens	$\bar{X} \pm SE$	No. specimens	$\bar{X} \pm SE$
Untreated control	74	173 ± 16	14	21 ± 1
O.v. infection and DMN treated				
No tumor developed	7	525 ± 180	2	500, 160+
Borderline	15	2,966 ± 1,317	3	500 ± 152
Tumor developed	41	4,950 ± 1,685	8	1,158 ± 415

*Quantitative analyses were carried out with diluted specimens (1:10 for serum and 1:20,000 for bile). The values shown had already been corrected for the dilution factor.

+Mean and SE were not calculated. The figures represent data from two animals.

elevated antigen levels when compared to the non-induction (untreated) controls. However, such an elevation was not as obvious. It is possible that the tumors would also develop in these animals if they were kept under observation for a few more months.

Our next question was how long it would take after DMN treatment before a noticeable elevation of antigen would be detectable. To determine this, groups of treated animals were sacrificed at various time intervals, as shown in Table 2. At each time interval, paired serum and bile specimens from the same animals were assayed and compared. In no instance did we find a serum antigen level to be higher than that of its paired bile specimens (Fig. 2). The data summarized in Table 2 show that there was a slight but noticeable elevation of the 200 kDa antigen in the bile even before DMN treatment was initiated. This finding was not altogether unexpected in view of the fact that bile duct proliferation and inflammation are known to occur during the very early stages of liver fluke infection[14] and that with opisthorchiasis there is a marked production and secretion of mucin, and possibly other proteins, by the bile duct epithelium. During the 10 weeks of DMN treatment, when tumors should not yet have developed (Thamavit, personal communications), there was already a several-fold increase of antigen levels in the serum and bile. In some animals, livers were sent for a microscopic diagnosis of CCA. Results presented in Table 3 clearly show that elevated serum antigen levels were more sensitive for detection of the early changes that take place during tumor development. However, once tumors had developed to the size that could be readily detected, either macroscopically or microscopically, there was no significant difference between the sensitivity for two methods. In addition to early detection, quantitation of the 200 kDa antigen level in serum has one additional advantage. That is, it could theoretically be used to follow progress or regression of tumor development. This conclusion is based on the data presented in Table 4. In this Table, only the data from tumor-bearing animals are analyzed and, as a group, a gradual elevation in antigen level was clearly demonstrated. When serial samples from a single animal were analyzed, a statistically significant elevation at each sequential time point was noted in a majority of the animals that subsequently developed a tumor. It should be mentioned also that 30-40% of the allotransplanted recipients exhibited elevated antigen levels in their sera.

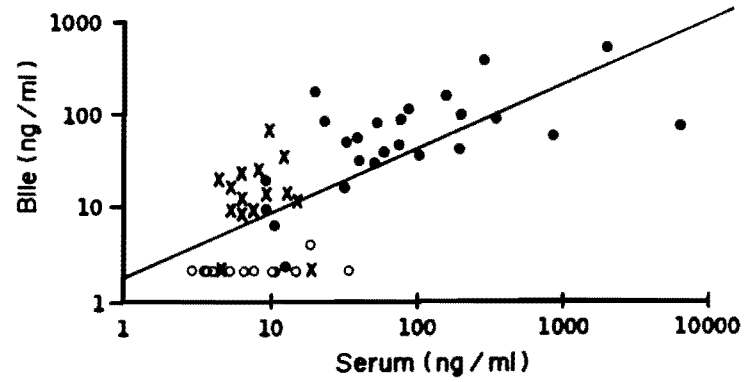

Fig. 2 Correlation of the antigen levels in paired bile and serum specimens from *O. viverrini*-infected (X), infected and DMN treatment (●) and untreated control (○) hamsters. The data plotted in the table represented the original uncorrected values from bile diluted 1:20,000 and serum diluted 1:10. A significant correlation ($r > 0.7$) of the antigen levels could be readily observed.

Table 2. Elevation of 200 kDa antigen during and following *Ov* infection and DMN treatment*.

Experimental groups	No. animals	Serum (ng/ml) $\bar{X}$ ± SE	Bile (ug/ml) $\bar{X}$ ± SE
Untreated control	14	103 ± 24	21 ± 1
After *O.v.* infection	15	89 ± 11	169 ± 41
During DMN treatment	11	606 ± 216	503 ± 157
Post DMN treatment	15	6,950 ± 4,187	1,244 ± 393

* See footnote of Table 1 for explanation.

Table 3. Correlation of histopathological changes and elevation of serum 200 kDa antigen in animals at different stages of tumor induction.

Time intervals after *O. v.* infection and DMN treatment	No. animals	No. with elevated serum level*	Histopathology		
			No tumor	Border line	Tumor
Immediately following DMN treatment	5 †	3 (60%)	5	0	0 (0%)
3-6 wk after DMN	13	11 (84%)	3	6	4 (31%)
8-10 wk after DMN	11	8 (73%)	3	2	6 (54%)
11-14 wk after DMN	17	14 (82%)	0	5	12 (70%)
16-18 wk after DMN	16	11 (69%)	0	2	14 (87%)
Total	62	47	11	15	37

* Compared with a cut-off level of X +2SD of 74 untreated controls (445 ng/ml).

† Figures represented the number of animals.

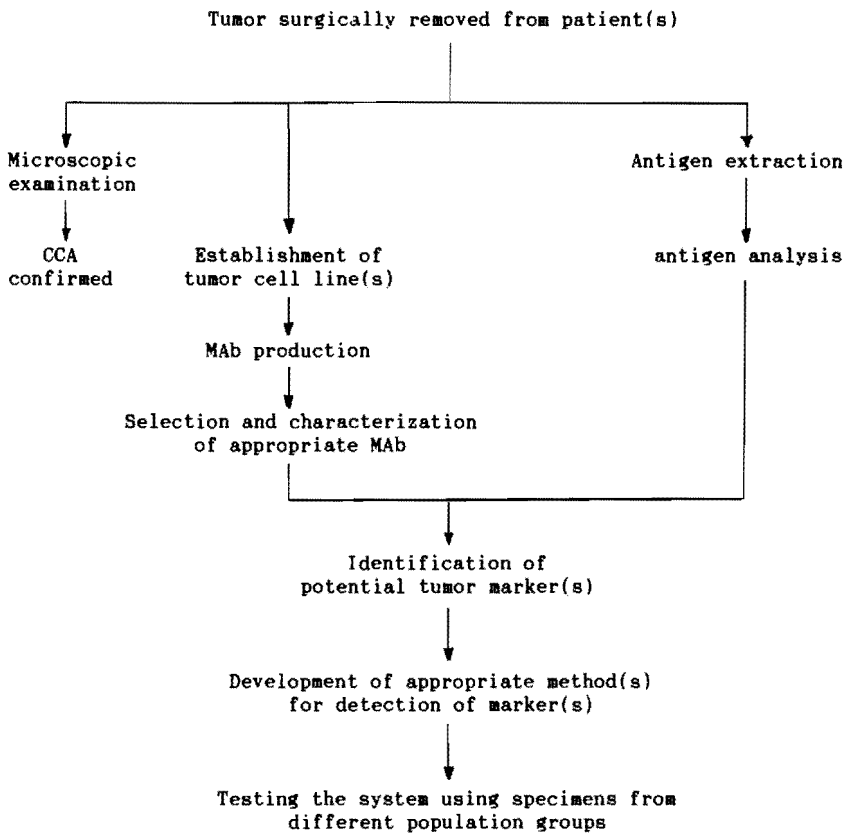

Fig. 3 Protocol for identification of tumor marker(s) with diagnostic potential for human cholangiocarcinoma.

Table 4. Kinetic study on the levels of 200 kDa antigen in serial serum samples from 40 tumor-bearing animals*.

Time intervals	$\bar{X} \pm SE$ (ng/ml)	Significant elevation†	
		No. animals	%
After *O.v.* infection (before DMN treatment)	341 ± 60	7	17
Immediately after DMN	863 ± 110	30	75
4 wk after DMN	903 ± 133	28	70
8 wk after DMN	1,195 ± 235	30	75
12 wk after DMN	1,755 ± 373	36	90

*Serial blood samples were taken form over 60 animals but only 40 animals had developed tumors at the time of sacrifice (judging from microscopic examination of the liver sections) and only the data from this tumor-bearing group of animals were used for calculation.
†Based on a cut-off level of $\bar{X} \pm 2SD$ from 74 untreated controls (445 ng/ml).

Progress in research on human cholangiocarcinoma

Results from the experimental animal studies are rather encouraging, as they suggest that it may be possible to obtain a similar end result with human cholangiocarcinoma. Therefore, a similar approach and/or protocol has been tried with humans. Some minor modifications (Fig. 3) were necessary because specimens from patients were not regularly available and were difficult to come by. Moreover, kinetic studies were not possible because, as mentioned earlier, serial withdrawal of blood from a large group of high risk individuals for an undefined period is neither practical nor easy to perform.

In order to obtain a sufficient quantity of tumor material to work with, we first attempted to establish tumor cell lines from patients with histopathologically proven cholangiocarcinoma. We were fortunate to obtain excellent cooperation from several groups of investigators and clinicians who kindly provided us with tumor tissues for the experiments. In our hands, it was not difficult to obtain primary cultures from these tumors, but it was very difficult to establish permanent cell lines from the primary cultures. After several attempts, we successfully established one cell line which has been maintained in our laboratory for more than 80 passages during the past three years.[10] Both light and electron microscopic features are similar to those of four hamster tumor cell lines (manuscript submitted for publication). The most notable ultrastructural characteristics are the presence of glycoprotein(s) or mucopolysaccharide covering microvilli of different densities, shapes and sizes, and intracellular lumens typical of adenomatous tissues. The cell line has retained its tumorigenicity as shown by its ability to propagate and subsequently develop into tumors in nude mice. It was also found to be immunogenic and to secrete a large number of soluble products. However, we were unable to detect any glycoprotein component that reacted with our MAb 6E5 against the hamster CCA. Instead, we found an interesting component in the supernatant fluids that migrated to a 160 kDa position and reacted with concanavalin A.

The secreted products were also analyzed for the presence of commonly known human tumor markers including CA 19-9, CA 125, CA 15-3, MCA (mucin-like carcinoma associated antigen), CEA and AFP. The only marker secreted in significant quantity was CA 125, but its concentration was only 10–20% of that secreted by pancreatic carcinoma cell lines (unpublished observations). However, trace amounts of MCA and CEA were also detected, particularly when the monolayers were cultured in the presence of serum supplement.

A mixture of the cell line homogenate and serum-free supernatant fluid was used for the production of MAbs in BALB/c mice. Among the several clones found to react with this antigen mixture by indirect ELISA, a few were found to react exclusively with the CCA antigen. By immunoblotting, these MAbs gave a few bands with crude CCA antigens and one of these was the 160 kDa glycoprotein secreted by the cell line. We are now in the process of developing a method for the quantitation of this antigen. It will most likely resemble the method described earlier for the hamster system.[12] We hope the tumors that develop in humans will behave similarly to those that occur in our animal model with regard to the production of tumor markers. We also hope that our approach with the animal model will work with

humans. If all goes well, we should be able to gather sufficient data on specific antigens for a final analysis and evaluation of their diagnostic potential within the next few years.

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