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Possibility of In-House Preparation of Liver Cancer Diagnostic Kits Based on AFP ELISA Test

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Abstract

Our research was focused on the study of possibility of preparation the AFP Enzyme-Linked Immunosorbent Assay (ELISA) diagnostic kits using tumor marker protein named alpha-fetoprotein (AFP) for hepatocellular carcinoma (HCC), the most frequent cancer in Mongolia. It is important to prepare the diagnostic kits for detection of liver cancer early, simply and inexpensively in this country. Detection of the marker in human sera would significantly help successful therapy, since the tumor could be tackled at an early stage in its development, often before metastases and other consequences. Therefore, level of AFP in sera would give possibility to control the progression of liver cancer. The micro titer plates were prepared for AFP ELISA diagnostic kits, and their sensitivity and specificity were 94.4% and 0.4, respectively. Statistically, there were no differences between our prepared micro titer plates for AFP ELISA diagnostic kits and commercially available ones ($t = 0.071$, $P = 0.94$, df = 24). It was concluded that there is a possibility to prepare AFP ELISA diagnostic kits using our prepared micro titer plates in house.

Key words: AFP, hepatocellular carcinoma, ELISA, kit

Introduction

Liver cancer is the sixth most commonly diagnosed cancer, and the third most common cause of death from cancer worldwide with incidence rate of 626,162 cases and mortality of 598,321 cases per year (Jeffrey *et al*., 2009 & http://www-dep.iarc.fr).

The incidence of HCC in Asia is high, particularly in Eastern and South Eastern Asia (Vanessa & Chung, 2007; Teo & Fock, 2001; David & Dirk, 2008). As it was reported by the International Agency for Research on Cancer, the highest rate of HCC occurred in Mongolia (Office for National Statistics, 2008; Welsh Cancer Intelligence and Surveillance Unit, 2008; ISD Cancer Information Programme, 2008; Northern Ireland Cancer Registry and Cancer Incidence and Mortality, 2008; http://www-dep.iarc.fr).

For instance, Oyunsuren *et al*. (2006) noted that 35.3% of all newly registered cancer cases were liver cancers, with an incidence rate of 51.3 per 100,000 populations in the period of 2000-2005, and the National Cancer Research Center of Mongolia reported that incidence rate of 54 per 100,000 populations in 2007 (National Cancer Research Center of Mongolia, 2007).

The main factors associated HCC are hepatitis C viruses (HCV) and hepatitis B viruses (HBV) infections, dietary aflatoxin B1 exposure and intake of alcohol in worldwide (Navas, 2007; Hwang & Hassan, 2009; Oyunsuren *et al*., 2006). Oyunsuren *et al*. (2007) defined most commonly occurring genotypes relating to hepatitis viruses in Mongolia: 1b genotype among HCV, D genotype among HBV and 1 genotype among HDV.

AFP is the most common used tumor marker for monitoring the response to therapy (usually chemotherapy) of HCC (Beastall *et al*., 1991; Aburano *et al*., 1980; Czauderna & Perilongo, 2004; Mashayekhi *et al*., 2005; Gerald, 2001).

Prior to metastasis, most cancers can be cured by localized treatments, such as surgery or radiotherapy. Unfortunately, about 70-80% of cancers has already metastasized by the time of diagnosis, and therefore, cannot be cured by surgery alone (Cooper, 1992).

The best approach to reduce the morbidity and

mortality from cancer is its prevention and early detection. Therefore, it is important to prepare the diagnostic kits for detection of liver cancer early, simply and inexpensively in Mongolia, where HCC incidence is very high. Besides of the diagnosis, the detection of AFP level in sera would help to control the stages and progression of liver cancer.

Materials and Methods

In total sera of 25 patients were used in this study, which diagnosed that 7 sera with liver cancer and 18 sera with chronic hepatitis or non liver cancer by the National Cancer Research Center of Mongolia. AFP ELISA diagnostic kits (Code: EMK 003), uncoated micro titer plates obtained from the Institute of Atomic Energy Beijing, China used for comparison. This kit consisted of a series of standard solutions of AFP at different concentrations from 10 ng/ml to 400 ng/ml, an enzyme conjugate, substrate solutions, reaction stopper, anti-AFP coated plate and buffered washing solution. Polyclonal antibody against AFP (82µg/ml) purified in house were used for coating process to micro titer plates.

Polyclonal antibodies were coated to micro titer plates by two methods (method I and method II) which differ from each other for dilution buffer, blocking solutions and experimental periods. The concentrations of antibody and blocking solution for coating procedures of ELISA were modified and diagnostic procedures was done as described (Alphafetoprotein (AFP) ELISA kit, My BioSource (Instrument)). Briefly, for preparation of micro titer plates of ELISA kit, each well of plates was coated with 4 µg/ml of antibody diluted in 0.1M $NaHCO_3$, pH 8.6 at 4^0C overnight. After rinsing, 3% BSA in PBS blocking buffer was added into wells and it was incubated at 37^0C for 1 hour. For the diagnosis, 50µl of standards, controls and sera were added per wells and incubated at 37^0C for 1 hour. 100µl of Conjugates were added after washing, and incubated at 37^0C for 1 hour. After washing, 100µl substrate solutions were added to wells and incubated 15 minutes at room temperature. Upon dropping 50µl Stop solution to each well, optical densities of reactants were measured by micro titer plate reader at 450 nm. Positive results were estimated that the level of AFP equals to 20 ng/ml or more.

The comparison of AFP binding capabilities of our prepared micro titer plates to commercially available kits was made by sandwich-ELISA and by constructing standard curve to determine AFP level in unknown sera. Our prepared micro titer plates were compared with imported commercially available ones by not only their AFP binding capabilities, but also their sensitivities and specificities. Statistical analysis of the data calculated with paired *t* test to compare AFP binding capabilities between imported commercially available micro titer plates and our prepared micro titer plates for AFP ELISA diagnostic kits.

Results

The values of optical densities (ODs) of AFP ELISA using micro titer plates prepared two kinds of method and commercially available ones were demonstrated in Table 1. Standard curves were drawn by using the ODs of each AFP standard (vertical axis) against the respective concentrations (horizontal axis) to support the determination of AFP level in the unknown sera (Fig. 1).

For computerized calculations and quality assessment normalized specific binding values, rather than OD values are used.

This standard curves indicate that optical densities (ODs) of micro titer plates prepared by method I were more taken in rather than method II. Thus, micro titer plates prepared by method I were preferred to appropriate to make AFP ELISA diagnostic kits for detection of marker protein named AFP of liver cancer. Furthermore, we purposed to compare the sensitivities and specificities of micro titer plates prepared by method I and imported commercially available ones for AFP ELISA diagnostic kits. Consequently, 25 sera were examined by sandwich-ELISA using the micro titer plates prepared by method I and commercially available ones (Table 2).

Table 2 demonstrates that 18 sera were normal or up to 20 ng/ml of AFP (negative), and 7 sera were abnormal or over 20 ng/ml of AFP (positive) of all 25 sera. Normal rate of AFP in adult human blood serum should be up to 20 ng/ml (Visual ELISA test for the qualitative determination of alpha-fetoprotein (AFP) in human serum (instrument); Enzyme immunoassay for the quantitative determination of AFP in human serum or plasma (instrument); Alpha-fetoprotein antibody, Rabbit IgG polyclonal antibody (description); Dominique *et al*., 1984; Kew, 1974; Johnson *et al*., 1978;

Table 1. The comparison of micro titer plates of AFP ELISA to create a standard curve

Samples		The micro titer plates prepared by Method I		The micro titer plates prepared by Method II		Micro titer plates of imported commercially available ones	
		OD at 450nm	B/B_{max}, %	OD at 450nm	B/B_{max}, %	OD at 450nm	B/B_{max}, %
Standards	A	0.054	6.32	0.054	10.32	0.058	4.04
	B	0.172	20.14	0.107	20.45	0.307	21.40
	C	0.320	37.47	0.186	35.56	0.521	36.33
	D	0.418	48.95	0.228	43.60	0.638	44.50
	E	0.696	81.50	0.393	75.14	1.147	79.98
	F	0.854	100.0	0.523	100.0	1.434	100.0
Negative control		0.116	13.58	0.087	16.63	0.087	6.06
Positive control		0.945	110.65	0.458	87.57	1.333	92.95

Specific binding value: B/B_{max},% = ($OD_{standard}$ /or $OD_{control}$/or OD_{sera})/($OD_{highest\ standard}$)x100%
For computerized calculations and quality assessment normalized specific binding values, rather than OD values are used.

Deyashiki *et al*., 1989). The values of B/B_{max},% = 20.14 (of micro titer plates prepared by method I) and B/B_{max},% = 21.40 (of micro titer plates of imported commercially available ones) were calculated using optical densities of 20 ng/ml standard solution (B) as presented in Table 1.

As stated in Table 2, sera examined in micro titer plates prepared by method I and calculated more than B/B_{max},% = 20.14 values were supposed to be positive (abnormal), less than B/B_{max},% = 20.14 values were supposed to be negative (normal).

Whereas, sera examined in micro titer plates using the commercially available ones and calculated more than B/B_{max},% = 21.40 values were supposed to be positive (abnormal), and less than B/B_{max},% = 21.40 values were supposed to be negative (normal) as well. For statistical assessment the results illustrated in Table 2 were calculated to expose the AFP binding capabilities of micro titer plates if there are some differences. As a result of paired *t* - test calculations two tailed *p* value equals 0.94, $t = 0.071$ and $df = 24$ (standard error of difference = 1.298). By conventional criteria, this difference is considered to be not statistically significant.

Sensitivities, which were equal to 94.4% of micro titer plates prepared by method I, 97.6% of imported commercially available ones and *specificities*, which were equal to same 0.4 of micro titer plates prepared by method I and commercially available ones, for AFP ELISA diagnostic kits,

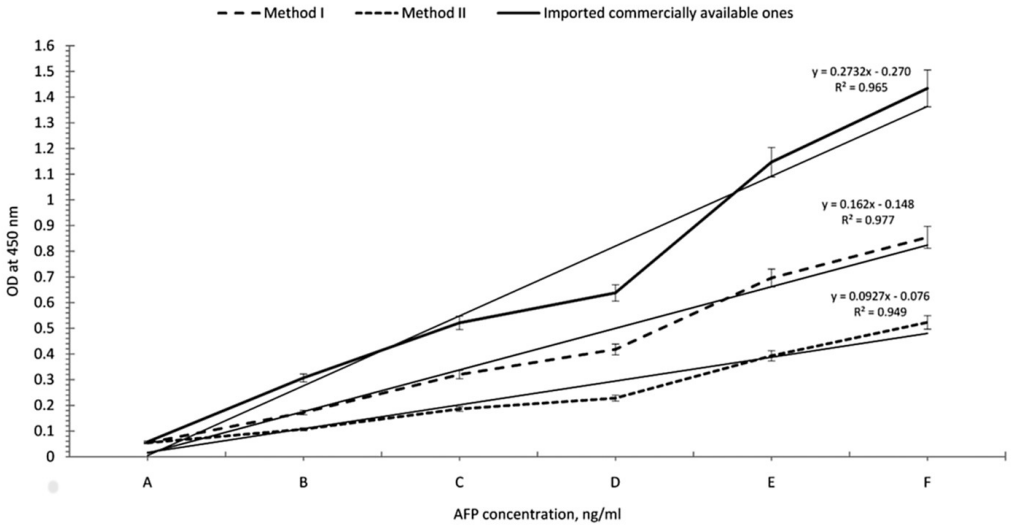

Fig.1. Standard curves of AFP level. AFP concentrations: 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml and 400 ng/ml are marked as A, B, C, D, E and F, respectively.

Table 2. Comparison of micro titer plates for their AFP binding capabilities

Samples (*n*=25)		The micro titer plates prepared by Method I			Micro titer plates of imported commercially available ones		
Gender	Age	OD at 450nm	B/B_{mx},%	AFP	OD at 450nm	B/B_{max}, %	AFP
female	65	0.124	14.52	-	0.184	12.86	-
female	45	0.085	9.95	-	0.211	14.71	-
male	-	0.062	7.26	-	0.199	13.88	-
male	33	0.056	6.56	-	0.163	11.36	-
female	48	0.062	7.26	-	0.237	16.53	-
male	39	0.07	8.2	-	0.168	11.71	-
male	30	0.104	12.18	-	0.193	13.46	-
male	43	0.093	10.89	-	0.146	10.2	-
female	31	0.127	14.87	-	0.298	20.8	-
female	77	0.099	11.6	-	0.206	14.36	-
female	67	0.211	24.7	+	0.391	27.26	+
female	68	0.117	13.7	-	0.209	14.57	-
female	64	0.166	19.44	-	0.203	14.15	-
male	-	0.153	17.91	-	0.206	14.36	-
male	82	0.144	16.86	-	0.199	13.87	-
female	42	0.077	9.02	-	0.125	8.71	-
female	-	0.063	7.38	-	0.079	5.5	-
male	45	0.093	10.89	-	0.212	14.78	-
male	-	0.640	74.94	+	0.886	61.78	+
female	62	0.715	83.72	+	1.020	71.13	+
female	52	0.517	60.54	+	0.733	51.11	+
male	47	0.227	26.58	+	0.321	22.38	+
female	69	0.687	80.44	+	1.029	71.76	+
male	58	0.129	15.1	-	0.245	17.08	-
male	49	0.321	37.59	+	0.736	51.32	+

were calculated.

Conclusion

The AFP ELISA system provides a direct quantitative determination of AFP level in human sera for diagnosis of HCC. AFP is a glycoprotein with a molecular mass of approximately 70 kDa. During fetal development, AFP maintains high level in sera and drops to very low level throughout the remainder of life. As a tumor marker, AFP has applications in primary liver carcinoma in adults and hepatoblastomas in children, in germ cell tumors and it is also occasionally elevated in cancer of the gastrointernal tract (Beastall *et al*., 1991; Alpha-fetoprotein (AFP) ELISA kit (instrument); AFP ELISA kit (instrument)).

The method to prepare micro titer plates of AFP ELISA diagnostic kits was elaborated to detect the level of AFP, liver tumor marker in human sera. The sensitivity of micro titer plates prepared by elaborated method was 94.4%, and the specificity was 0.4, which indicate possibility to apply our prepared micro titer plates for HCC diagnostic procedure. In addition, there were no significant statistically difference between our prepared micro titer plates and imported commercially available ones to determine the AFP level in human sera (t = 0.071, p=0.94). Furthermore, it would be possible to prepare internal products using our prepared micro titer plates for AFP ELISA diagnostic kits.

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