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A Study on the Analysis of a New Broad Spectrum Tumor Marker (GIFTEC Reagent)

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The GIFTEC test is a very convenient test to detect the cancer within 30 minutes by using random urine specimens of various types of cancer. This new marker is appropriate for cancer screening. However, some problems have been pointed out, such as the high rate of false positive reaction by benign diseases, low sensitivity and unelucidated reaction material. The pupose of this study is to analysis the reaction material by fractionation and examine the clinical usefulness of the GIFTEC test, as compared with the usefulness of the polyamine test The salting-out process was conducted with 10% and 20% ammoium sulfate, and gel filteration was conducted with Sephacryl S100 with the low pressure pump to compare changes in elution pattern. Then, the GIFTEC test was taken with the GIFTEC kit, simultaneously with the polyamine test with the enzyme immunoassay. Pellet was not separated with the ammonium sulfate. But after the gel filteration, the specificity and diagnostic efficiency of the GIFTEC test rose from 80.2% to 97.1%, and from 79.6% to 94.5%, respectively, though its sensitivity remained at 65.6%. As a result, its false positive rate could be lowered and its diagnosibility could be raised. The diagnostic efficency of the GIFTEC test was superior when used in combination with the polyamine test. The GIFTEC test after gel filteration has higher diagnostic efficiency and lower false positive reaction rate, and it has been found to be more useful than the polyamine test. Thus the GIFTEC test can be a useful tool for screening early cancers due to its advantages like rapidity, simplicity, low cost and use of random urine. If used in the form of combination assay with the polyamine test, the GIFTEC will be particularly useful in discriminating the normal group from the high risk group.

Key Words: Fraction, Gel filteration, GIFTEC test, Polyamine test

INTRODUCTION

The shortcut to conquering cancer is still its early detection and operation, and the development of methods of diagnosing cancer early is surely among the most important issues in modern medical science. Polyamine,[1~3] TPA,[4] CEA[5] beta 2 microglobulin,[6] and fibronectin[7] are commonly used as broad spectrum tumor markers for early diagnosis and screening of cancer through urine, but their clinical utilities are relatively low because of their low specificity, use of 24h urine, complexity

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of testing procedures, and confusion in result interpretations. But the GIFTEC has been recognized as a very useful marker because it can detect parahydroxyphenyl-derivatives of urine in all kinds of cancer, as reported first by Son, Kim, and the research team of Sam-Il Pharmaceutical, Inc.[8,9] in 1988, and as tested later in many clinical experiments.[10~14]

The GIFTEC is a diagnostic reagent for screening which detects cancer by checking parahydroxy phenyl-derivatives in the urine. The metabolites produced in the GIFTEC reaction exit as hydroxyphenyl-derivatives whose positions 3 and 5 can not be replaced, which consists of peptides containing tyrosine and tyrosin at the end and catecholamine metabolites containing tyrosine at the start. Accordingly, this test can detect cancer through urine specimen of unspecified cancer cases. It also has other advantages: use of random urine; prompt test results (less than 30 minutes); and simple testing and reading procedures. The GIFTEC has proven to be very useful for screening in cancer diagnosis. However, some problems have been pointed out in its clinical applications: unelucidated response material; and high false positive rate. Polyamine, like spermidine, spermin, and putrescine, is a positive ion in the cells of low molecular weight, and it is involved in the control of RNA-dependent protein synthesis and essential in cell proliferation and division.[2,15,16] Its biosynthesis and accumulation increases rapidly with proliferation of cells as in cancer, and the density of polyamine in cancer cells is higher than that in normal cells. Since it was reported that polyamine level increases in the urine in patients with cancer,[17] and also in their serum[3] and CSF,[18] polyamine has been noted as a useful cancer marker.

Accordingly, in order to raise the sensitivity and selectivity of response material in the GIFTEC test, we have conducted fraction analysis by means of salting-out by ammonium sulfate and gel filteration and investigated the structure of parahydroxyphenyl derivatives and compared the diagnostic efficiency. On the basis of the results of this analysis, we have also conducted combination assay with the polyamine test in order to compare diagnostic efficiency and usefulness of both tests.

MATERIALS AND METHODS

1) Subjects and specimens

The subjects used in this study consisted of 350 healthy persons who took medical checkups at the Health Center of the Pusan National University Hospital during the period of May through November 1996, and 32 patients who were hospitalized in Pusan National University Hospital and histopathologically diagnosed as cancer during the same period. Those who skeptically had other diseases or showed brown color in the GIFTEC test were excluded. Random sampled urine was used as specimen in the experiment. The specimen was preserved in the frozen state below -20°C when it was not used immediately after collection.

2) Methods

(1) GIFTEC test: As proposed in Son,[11] 5 ml of urine specimen was put in the test tube, and 0.6

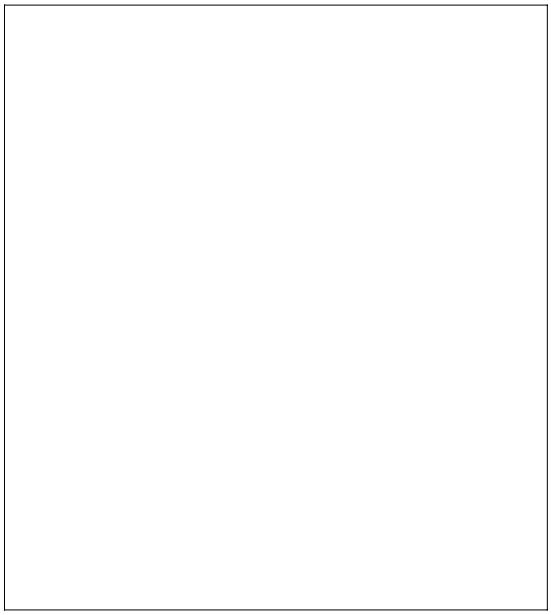

Fig. 1. Method of GIFTEC Test.
* Delta OD = Abs. of tube 1 - Abs. of tube 2

ml of precipitating reagent was added. Then, it was put for reaction in the waterbath of over 90°C for ten minutes, and frozen immediately. After 5-minute centrifugation at 3000 rpm, 1 ml of color reagent was added for reaction with the supernatant, and then it was left for 10 minutes. The difference (Delta OD) was measured between the absorbance of the specimen with color reagent (Sample OD) and that with distilled water instead of color reagent (Blank OD) at 490 nm (Fig. 1).

(2) Spectrum checking: Absorbance of each wave of specimen after reaction was measured with distilled water as base by Shimazu 150 spectrometer from 1,000 nm to 330 nm.

(3) Salting out with ammonium sulfate: For salting-out of 10% ammonium sulfate, 120 ml of urine was adjusted to pH 3 and separated centrifugally at 3,000 rpm for 10 minutes. 100 ml of the supernatant was was moved into a beaker on the ice, and 5.5 g of ammonium sulfate was added and melted in it. Then it was divided units of 50 ml, and each unit was separated centrifugally at 7,500 rpm for 25 minutes. Together with 2 ml of its supernatant, the pellet melted into 2 ml of distilled water was used in the GIFTEC test. The above procedure was repeated with 20% ammonium sulfate made with mixture of 98 ml (10%) of the supernatant and 5.5 g of ammonium sulfate.

(4) Fraction by gel filteration: Buffer was made of 0.2M phophate solution and 1% NaN_3, and was used as stock and loading buffer. Sephacryl S-100 in gel state made from 20% ethanol is packed in column. The urine specimen is distilled with the 0.45 μm pore and concentrated with the centriplus. For fractionation, a colum of 2.8 cm diameter and 80 cm length is prepared, and Sephcryl-s100 after degassing and swelling with 0.01M sodium phosphate buffer (pH 7.4) is charged. After steady state with buffer, 1 ml of specimen was infused and gel filteration was conducted at the pump speed of 0.5 ml/min and 1 ml/min. The GIFTEC test was taken of each separated fraction.

(5) Polyamine test: The polyamine of urine was measured with a polyamin test kit (TOKUYAMA Soda Co. Japan) which uses the principle of EIA devised by Kubota et al.[19] The instructions in the kit were followed, and after reaction, absorance was measured at 510 nm with a spectrophotometer, and the measured amount of creatinine was converted into polyamine density, which was indicated as umol/g creatinine. The cut-off value of the urine polyamine was kept less than 40 umol/g creatinine.

RESULTS

Table 1. Results of GIFTEC test by salting-out with ammonium sulfate

After Sample	Before	10%		20%	
		Supernatant	Pellet	Supernatant	Pellet
1	1.091	1.002	0.333	0.809	0.292
2	1.142	0.469	0.230	0.375	0.152
3	0.862	0.841	0.320	0.717	0.297
4	3.051	2.986	0.336	1.801	0.261
5	1.381	1.347	0.147	1.0	0.065
6	1.309	1.263	0.305	0.858	0.239
7	1.063	1.027	0.247	0.695	0.128
8	1.979	1.384	0.210	0.774	0.154
9	1.567	1.489	0.289	1.003	0.161
10	1.204	1.13	0.345	0.928	0.140

Fig. 2. Representative chromatogram of urine in patients with heaptocellular carcinoma. After GIFTEC test for each fraction, pinkish color is noticed on fraction of 17th tube (Fraction time: 5 min, chart speed: 1 mm/min, pump: 1ml/min and absorbance range selector: 0.5).

Fig. 3. Representative chromatogram of urine in case of normal healthy subjects. After GIFTEC test for each fraction, pinkish color is noticed on fraction of 41st and 47th tube (Fraction time: 5 min, chart speed: 1 mm/min, pump: 0.5 ml/min and absorbance range selector: 0.5).

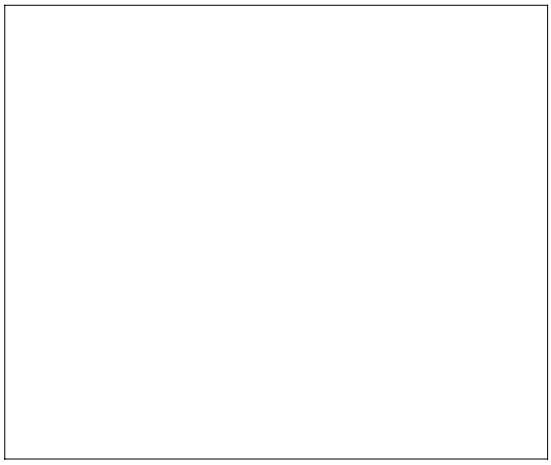

Fig. 4. Representative chromatogram of urine in patients with cancer. After GIFTEC test for each fraction, pinkish color is noticed on fraction of 34th tube (Fraction time: 5 min, chart speed: 1 mm/min, pump: 0.5 ml/min and absorbance range selector: 0.5).

1) GIFTEC test results after ammonium sulfate treatment

Table 1 shows the results of the GIFTEC test after ammonium sulfate treatment of 10 samples of positive specimens whose delta OD was 0.5 or higher at the pre-GIFTEC test for cancer patients. As can be seen in this table, in all cases, the pellet shows negative response at the GIFTEC test, and in 9 of 10 cases, the supernatant shows positive response both before and after the salting-out process with 10% and 20% ammonium sulfate. Since the pellet showed no meaningful response, this method was abandoned as useless.

2) Gel filteration

Fig. 2 shows the chromatogram at the pump speed of 1 ml/min. As we can see in this Figure, the peak of each fraction is not clear. Fig. 3 shows the results of the fraction test for normal person who took health check-ups and showed high OD at the GIFTEC test. This Figure shows that healthy persons show false positive response in the fractions of 30's and 40's, whereas in the urine of cancer patients, the positive response of the GIFTEC test in 30's (Fig. 4).

Table 2 shows the positive response of each fraction after the gel filteration for 69 (19.7%) of 350 healthy subjects and 21 (65.6%) of 32 cancer patients.

As we can see in this table, all cancer cases have the positive fraction, and 59 of 69 healthy cases with false positive response show the negative

Table 2. Results of GIFTEC test before and after gel filteration

Subject	Number	Positive GIFTEC test	After gel filteration
Normal	350	69	10
Cancer	32	21	21
Total	382	90	31

Table 3. Diagnostic effciency of GIFTEC test before and after gel filteration

Subject₩Gel filteration	Before	After
False negative	11	11
True positive	21	21
True negative	281	340
False positive	69	10
Sensitivity	65.6%	65.6%
Specificty	80.2%	97.1%
Diagnostic efficiency	79.6%	94.5%

fraction response or overlapped positive response even after positive response and can be easily distinguished. Through this process, we can possibly reduce false positive rate. However, further research is needed on positive cases for their latent cancer or false positive response.

3) Diagnostic efficiency of the GIFTEC test before and after gel filteration

Table 3 shows the diagnostic efficiency of the GIFTEC test before and after the gel fileration for 350 healthy subjects and 32 cancer patients. It should be noted in this table that after the gel fileration, the specificity increased and diagnostic efficiency rised, though the sensitivity remained unchanged due to the decrease in false positive. Therefore, it follows that the gel fileration can possibly be used as a test to check false positive response when specimens have shown positive response at the first screening.

4) Diagnostic efficiency of the GIFTEC test and polyamine test

Table 4. Diagnostic efficiency of GIFTEC test and polyamine

Subject	GIFTEC	Polyamine
False negative	11	13
True positive	21	19
True negative	340	261
False positive	10	89
Sensitivity	65.6	59.3
Specificty	97.1	89.1
Diagnostic efficiency	94.5	91.4

Table 5. Combination assay with GIFTEC anf polyamine test

Tumor marker	Positive cases	Positive rate (%)
GIFTEC alone	21/32	65.6%
Polyamine alone	19/32	59.3%
Either GIFTEC or polyamine	24/32	75.0%

The GIFTEC test shows 65.6%, 97.1% and 94.5%, and the polyamine test 59.3%, 89.1% and 91.1&, respectively in sensitivity, specificity and diagnostic efficiency (Table 4). This result means that the GIFTEC test is superior to the polyamine test in diagnostic efficiency.

5) The combination assay of the GIFTEC test and the polyamine test

Table 5 shows the positive response rate of cancer patients when the combination assay of both GIFTEC and polyamine tests is used. When the two tumor markers are used separately, their positive response rate is 65.6% for the GIFTEC test, and 59.3% for the polyamine test. But when they used in combination, it has risen to 75.0% (24 of 32 cancer cases showed positive reponse).

DISCUSSION

In Korea, over 50,000 persons (0.1% of the population) die of cancer, while in the U.S., over 30,000 persons die only of prostate cancer, every year. The order of frequency in Korea is stomach

cancer, lung cancer, and hepatoma for male, and uterine cancer, stomach cancer, breast cancer for female, whereas in the U.S., lung cancer, prostate cancer, colon cancer for male, and lung cancer, breast cancer, and colon cancer for female. Recently Korea is gradually following the Western pattern in that lung and breast cancers are increasing. In spite of all the modern advanced means of medical treatments, metastatic cancer is still difficult to cure. It is most important to diagnose and treat cancer early before its distant metastasis. Hence we need a good test which can detect cancer early in various organs of an unspecified human body with high degree of precision and also with high rate of discrimination for negative cases. Broad spectrum tumor markers can be used for this purpose. One of them is the GIFTEC test, which is convenient, easily available, and diagnostically efficient. It is for an early diagnosis of cancer using urine. After checked at the fact that spectrum can be found through NMR in the urine of cancer patients, Son, Kim & Research Lab of Samil Pharmacetics, Inc.[8,9] conducted biochemical analysis of its material, and devised the test. The test is very useful in health check because it can tell cancerous from noncancerous disease and find out a risk group with high degree of sensitivity. Similar results are reported in Cho and Kim,[13] and in the cases of gastro-intestinal disease[10] and of obstetric disease.[14] However, some of the reports[8~14] point it out that the GIFTEC test has problems in screening cancer because it also shows high positive rate in the cases of liver disorder, renal disorder, diabetic disorder, and drug disorder, etc. One of the best ways of minimizing the false positive rate is to analyze material precisely. There are various ways of separating protein involved in antigen-antibody reaction, but this study has adopted the salting-out process with high salt together with the gel filteration to investigate the material of the positive fraction at the GIFTEC test. The process of melting protein with high salt is most commonly used in enzyme purification. In this study, the salting-out method was tried with 10% and 20% of ammonium sulfate, but it could not be used because it did not react to pellet. There may have been some problems with the extraction method, but the salting-out method could not be adopted because no response material could be found even with saturated concentration of 10%. In this study, positive response material could be found in the fraction of 40's in the process of investigating each fraction through the gel filteration using a lower pressure pump. But it was found that the speed of a pump could affect the sensitivity of fraction. Smaller quantity per minute could make a clear distinction beween peaks, and the speed of 0.5 ml/min was believed to be appropriate. And the false positive material of healthy persons tends to show two positive fractions and it is expected to be clearly distinguished from positive urine of cancer patients. This needs further research. Continous observation of positive reaction cases will raise the reliability of results. This fraction test is undesirable in that the process takes a long time, but it has made it possible to get a large quantity of positive reaction material. The material could possibly be used in producing sequence or antibody. If we collect and concentrate peak fractions made this way and conduct SDS-PAGE electrophoresis, comassie brilliant blue dying, and silver stain on them in order to improve their sensitivity and specificity, we can analyze their pattern. This also needs further research. This study does not cover cases of positive disorders, and can not be compared with other studies. But it shows that after the gel filteration, the specificity and diagnostic efficiency of the GIFTEC test rose from 80.2% to 97.1% and from 79.6% to 94.5%, respectively, though its sensitivity remained at 65.6%. Hence the GIFTEC test is believed to be very useful in identifying false positive reaction after the first screening of cancer. A combination of tumor markers can raise true positive rate by reducing false negative rate in cancer screening, and this method has been called as “combination assay” or “cancer profile”.[22~24] Sintsura and Karada[23] say that positive rate in the diagnosis of pancreas cancer could be raised to 92% by means of the

combination assay of ferritin, RNase, CEA, and trypsin, and that false negative rate can be reduced over a wide range of diseases through a combination of independent markers. They also argue that unlimited increase in the number of markers may cause dangerous increase in false positive rate, and suggest that it is appropriate to combine two markers. Since polyamine, the positive ion of low molecule involved in protein synthesis, was first reported to be useful in screening cancer,[1,25] it has been repeatedly reported to increase in various kinds of cancer.[26~28] This study shows that the sensitivity and diagnostic efficiency of the polyamine test alone were 59.4% and 91.4% respectively, being lower than those of the GIFTEC test taken at the same time. Ployamine's sensitivity for cancer diagnosis is lower in this study than that reported in Yang et al,[29] supposedly due to the difference in cut-off level. This matter can be confirmed by increasing the number of specimens. This study has confirmed that the combination assay of both GIFTEC and polyamine tests raised diagnostic sensitivity to 75.0%. To get better results in cancer diagnosis, it is strongly recommended that the GIFTEC test be used in cambination with other broad spectrum tumor markers like polyamine. In screening cancer, the test through tumor markers requires high sensitivity in cancer patients and high specificity in non-cancerous benign diseases. In other words, it requires high true positive ratio and low false positive rate. Such an ideal combination assay of tumor markers could make early diagnosis of cancer possible.

To summarize, false positive reaction, which has been pointed out as the primary problem of the GIFTEC test, can be identified to a certain degree through fraction analysis, and the GIFTEC test is recommended to be taken (1) simultaneously with other broad spectrum tumor markers at the first stage of screening, and then (2) in the form of combination assay with tumor-specific antigen to the organs of the human body in the order of incidence rate, only for the positive cases of the first stage. In that way, the GIFTEC test may be very useful in the cancer screening, that is, in the risk group detection. The problems of brown-colored urine. false positive reaction caused by other factors, and false negative reaction are left for future research.

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