African Scientist Vol. 24, No. 1 March 31, 2023
Printed in Nigeria

1595-6881/2023 $80.00 + 0.00

https://africansciientistjournal.org

AFS2023013/24119

Arachis hypogaea Seeds Reduced Glucose Metabolism in Colon of Rats Exposed to 1,2-Dimethylhydrazine

Isoje, Abigail Okezi[1*] and Obi, Frederick Otunuya[1,2]

[1]Department of Biochemistry, Faculty of Life Sciences, University of Benin, Edo State, Nigeria
[2]Department of Biological and Chemical Sciences, Faculty of Natural and Applied Sciences, Michael and Cecilia Ibru University, Agbarha –Otor, Delta State, Nigeria

*Corresponding author Email: isojeabigail@gmail.com, Tel: +234 803 255 3611

(Received March 27, 2023; Accepted in revised form March 30, 2023)

ABSTRACT: The aim of this study was to ascertain the effects of peanut diet supplementation on glucose metabolism in rats exposed to 1, 2-dimethylhydrazine. Lactate dehydrogenase and glucose -6-phosphate dehydrogenase activities were used as indices for glucose metabolism. Eighty-four rats of both sexes were used for this study and were divided into seven groups of 6 rats each. 1, 2- dimethylhydrazine (DMH) was administered subcutaneously at a dose of 25 mg/kg body weight. Group A rats were maintained on rat feed, group B rats were maintained on rat feed and administered DMH once weekly for 12 weeks. The group C rats were administered DMH weekly for 24 weeks while on normal diet. Group D rats were administered DMH and feed for 12 weeks followed by peanut supplemented diet for the next 12 weeks. The group E rats received DMH weekly and peanut supplemented diet for 24 weeks while group F rats had peanut supplemented diet for 12 weeks before the administration of DMH for 12 weeks. Group G rats were maintained on only peanut supplemented diet for 24 weeks. Relative to the control and diet supplemented groups, Lactate dehydrogenase and Glucose-6-phosphate dehydrogenase activities were significantly ($p \leq 0.05$) increased in the colon of the DMH-only treatment group. Thus, increased glucose consumption occasioned by the exposure to DMH can be altered by the peanut supplementation in the diets of exposed rats.

Keywords: 1,2-Dimethyhydrazine, Glucose metabolism, Lactate, *Arachis hypogaea*, Colon carcinogenesis

Introduction

The fact that malignant cells produce increased amount of lactate compared to normal cells in the presence of oxygen, signifying more glucose utilization than normal cells, has been established (Hagland and Søreide, 2015). This discovery referred to as the "Warburg effect" was previously thought to be as a result of hostile tumour microenvironment and attendant dysfunctional mitochondria, leading to an increase in glycolysis (Moreno- Sanchez *et al*., 2007). This theory however was later found not to be true, as majority of malignant cells still possess functional mitochondria and utilize oxidative phosphorylation and glycolysis for energy extraction from nutrients (Moreno- Sanchez, 2007; Ward and Thompson, 2012). All the same, the finding that there is increased glucose flux during malignancy remains true and is utilized in 2-fluorodeoxyglucose positron emission tomography (2-FDG PET) scan for evidencing metastasis in clinical practice (Hagland and Søreide, 2015). The choice of glucose as preferred energy source is currently recognized as a major feature of malignancy (Hanahan and Weinberg, 2011). The increase in the breakdown of glucose is essential for sustaining rapid cell growth and increase in terms of steady energy supply, and provision of other growth facilitators associated with glucose catabolism in the pentose phosphate pathway (Hagland and Søreide, 2015). The latter involves the enzymatic change of glucose-6-phosphate and fructose-6-phosphate to ribose-5-phosphate, required for nucleotide production. In addition, is glyceraldehyde-3-phosphate, a starting material for the phospholipids required for the formation of new membranes. This adjustment in metabolism by malignant cells is required to ensure that the high rate of proliferation is sustained (Hagland and Søreide, 2015).

It is obvious that rapid glucose metabolism is a mechanism by which malignant cells sustain themselves, and studies have shown that the consumption of peanut supplemented diet reduces 1,2- dimethylhydrazine-induced colon carcinogenesis in rats. The purpose of this study was therefore to ascertain whether the consumption of peanut supplemented diet will alter glucose metabolism.

Materials and methods

Plant material: Arachis hypogaea seeds were purchased at Uselu market, Benin City. The verification of the peanut seed and plant was carried out by Dr. Henry A. Akinnibosun of the Department of Plant Biology and Biotechnology, University of Benin. The peanut plant was then deposited at the herbarium and assigned a voucher number UBH_A352.
Chemicals: The following analytical grade reagents were used for this study; 1,2-Dimethylhydrazine (DMH) dihydrochloride (Sigma Aldrich, Germany), halothane (Piramal Healthcare Limited, India). All other chemicals not itemized were also of analytical grade.
Animals: A total number of 84 healthy male and female albino rats (Wistar strain) were used for this study. They were purchased from Department of Biochemistry, Animal Unit, Faculty of Life Sciences, University of Benin, Benin City Nigeria and housed in wood framed/iron meshed cages in the Animal House of Biochemistry Department. The rats were acclimatized for 14 days prior to the commencement of the experiment. During the acclimatization period the rats had unrestricted access to feed (Grow ers mash, Bendel Feeds and Flour Mills Ltd. Ewu, Edo State, Nigeria) and water.
Rat diet formulation: The groundnut was incorporated into the rat diet at 20 % level (Guyton *et al*., 2008).
Prepartaion of 1,2-dimethylhydrazine (DMH) dihydrochloride solution: Stock DMH was dissolved in 1 M ethylenediaminetetraacetic acid (EDTA-disodium salt)-saline solution, pH 6.5, just before use. The adjusted pH was to ensure that the carcinogen was stable at the time of administration.
Animals grouping and treatment: The rats were divided into seven groups of 6 male rats each and another 7 groups of 6 female rats each. DMH was administered at a dose of 25 mg/kg body weight subcutaneously (Veceric and Cerar, 2004). Group A (control) rats of either sex were maintained on normal feed (growers mash) and a weekly subcutaneous injection of the vehicle (EDTA-Saline). Group B rats were provided normal rat feed and injected with DMH once weekly for 12 consecutive weeks. Group C rats were placed on normal feed and injected DMH once weekly for 24 weeks. Group D rats received DMH and normal feed for 12 weeks followed by the peanut diet (at 20% level) for the next 12 weeks. Group E rats received DMH and peanut diet (20% level) concomitantly for 24 weeks. Group F rats were placed on peanut diet for 12 weeks prior to the administration of DMH for 12 weeks. Group G rats were placed on 20% peanut diet throughout the experimental period of 24 weeks, but received once weekly injection of the vehicle (EDTA-saline). All groups were kept on their respective diet for 24 weeks.
Collection and preparation of samples for analyses: The study was carried out with strict compliance with the ethics in Guidelines and Specification on Experimental Animal Care (Animal Care and Use Program, 2011). Rats in each group were sedated with halothane. While under anaesthesia the abdominal and thoracic regions were opened and blood collected into plain sample tubes by heart puncture. The colon was excised. Sections of the colon were obtained for biochemical assessments carried out on the homogenate supernatants. The colon and liver homogenates of each rat were prepared by grinding 1 g in ice-cold mortar with acid-washed sand in 5 mL physiological saline solution. Each homogenate was subjected to centrifugation at 3500 rpm for 5 minutes and a clear supernatant obtained which was kept at -20 °C until needed.
Biochemical assays: The estimation of the concentration of total protein in the tissue homogenate supernatants was done using the Biuret method. The activity of lactate dehydrogenase (LDH) was determined using the UV absorbance - based method which is an optimized standard method according to the recommendations of the Deutsche Gesellschaft für Klinische Chemie (1970). Glucose-6-phosphate dehydrogenase activity was estimated using the UV absorbance-based method of Lohr and Waller, (1974) in which the rate of change in absorbance at 340 nm due to the reduction of $NADP^+$ was the basis for determining the activity of the enzyme.

Results

Consumption of peanut diet before the administration of DMH (Group F) significantly ($p \leq 0.05$) improved weight gained by these rats relative to the group of rats that received DMH only for 12 weeks and maintained on normal diet (Group B) (Table 1). Rats that were administered DMH and consumed peanut supplemented

diet concomitantly (Group E) had significantly ($p \leq 0.05$) increased weight gain when compared to Group C rats ,that had weekly injection of DMH for 24 weeks but maintained on normal diet. In the female rats, weekly subcutaneous injection of DMH for 12 and 24 weeks respectively resulted in significant ($p \leq 0.05$) decreases in weight gain relative to the corresponding control group (Table 1). Exposure to DMH only for 24 weeks (Group C) significantly ($p \leq 0.05$) decreased weight gain relative to the group of rats exposed to weekly administration of DMH for 12 weeks (Group B).

The results of colon glucose-6-phosphate dehydrogenase activity assays of male and female rats are presented in Table 2. The activities of the enzyme in the colon of male and female rats administered DMH only for 12 and 24 weeks respectively were significantly ($p \leq 0.05$) increased relative to the activity in appropriate control group. Supplementation with peanut diet 12 weeks after the administration of DMH for 12 weeks (Group D) significantly ($p \leq 0.05$) lowered the activity of the enzyme relative to the groups that received DMH administration for 12 and 24 weeks respectively but maintained on the normal feed (Groups B and C). Concomitant administration of DMH and consumption of diet supplemented with peanut for 24 weeks (Group E) and the consumption of the supplemented diet for 12 weeks before the administration of DMH for 12 weeks (Group F) caused significant($p \leq 0.05$) reduction in the activity of glucose-6-phosphate dehydrogenase.

Male and female rats that consumed peanut supplemented diet for 24 weeks without the administration of DMH (Group G) had glucose-6-phosphate dehydrogenase activities that were comparable to those of the control groups.

Results of colon total protein level analysis for male and female rats are presented in Table 2. The level significantly ($p \leq 0.05$) reduced in the group that received DMH for 12 and 24 weeks respectively in both male and female rats relative to control. Consumption of peanut supplemented diet for 12 weeks before the administration of DMH (Group F) and concomitant consumption of the supplemented diet along with DMH administration for 24 weeks (Group E) significantly ($p \leq 0.05$) increased the total protein level compared to the levels in both male and female rats treated for 12 and 24 weeks respectively with DMH only. In the female rats, consumption of peanut supplemented diet after exposure to DMH for 12 weeks significantly ($p \leq 0.05$) increased the total protein level relative to the group that received DMH for 12 weeks and maintained on normal rat feed (Group B). In the corresponding male group however, the increase was not significant.

The results of the assay of lactate dehydrogenase activities in colon of male and female rats are presented in Table 2. The enzyme activity significantly ($p \leq 0.05$) increased in DMH only treated groups, relative to the control group in both male and female rat colon. In both male and female rats, consumption of peanut supplemented diet after 12 weeks of DMH treatment also significantly ($p \leq 0.05$) reduced LDH activity relative to the group that received DMH for 12 weeks and maintained on normal diet.

Table 1: Effect of Peanut supplemented diet on weight of DMH-treated rats

Group	Treatment	Rats (Males)			Rats (Females)		
		Initial weight	Final weight (g)	Weight gain	Initial weight	Final weight (g)	Weight gain
A	Control	116.00±5.29	231.50±10.10	115.00±41.98	101.60±2.30	180.80±7.98	79.75±10.10
B	T_{12}/N*	111.50±4.73	203.25±25.05	74.50±16.34**	100.80±1.79	178.40±29.15	76.75±19.05
C	T_{24}/N	117.50±2.73	210.25±20.79	90.25±46.73	120.80±8.53	156.20±9.28	38.50±17.13^{a,b}
D	T_{12}/P_{12}	120.00±5.09	246.00±33.32	131.50±30.70	105.40±2.19	156.60±26.00	48.50±29.40^{a,b}
E	T_{24}/P_{24}	120.00±5.10	270.75±30.93	152.00±66.37^{b,c}	110.00±5.29	167.00±39.80	74.00±20.93^{c}
F	P_{12}/T_{12}	116.25±10.90	250.75±25.90	162.75±30.88^{b,c}	108.80±4.44	212.40±16.39	110.00±18.97^{c,d,e}
G	P_{24}	101.00±0.00	246.75±17.07	146.75±46.56^{b}	100.00±0.00	178.80±16.33	77.50±25.55^{c,f}

*Control = Normal rat chow (N) and water with weekly subcutaneous injection of EDTA-saline solution.

T_{12}/N = Maintained on normal chow (N) while treating with 1,2-dimethylhydrazine (DMH) for 12 weeks.

T_{24}/N = Maintained on normal chow but treated with 1,2-dimethylhydrazine (DMH) for 24 weeks.

T_{12}/P_{12} = Treated with DMH for 12 weeks while on normal rat chow and later maintained on peanut diet (P) for12 weeks.

T_{24}/P_{24} = Treated with DMH and peanut simultaneously for12 weeks.

P_{12}/T_{12}= Maintained on peanut diet for 12 weeks, followed with DMH treatment for 12 weeks.

P_{24} = Maintained on peanut diet for 24weeks with weekly subcutaneous injection of EDTA- Saline solution.

**Values with superscripts a, b, c, d, e or f are significantly different from the value of the group with the corresponding upper case letter A, B, C, D, E or F ($p \leq 0.05$).

T represents DMH.

Table 2: Effect of Peanut supplemented diet on colon total protein levels, glucose-6- phosphate dehydrogenase and lactate dehydrogenase activities of DMH-treated rats

Group	Treatment	G6PD Activity (µm/mL colon homogenate supernatant) Mean ± SD×10^2 (n = 4)		Colon Total Protein (g/dL colon homogenate supernatant) Mean ± SD (n=4)		LDH Activity (U/L colon homogenate supernatant) Mean ± SD ×10^2 (n = 4)	
		Rats (Male)	Rats (Female)	Rats (Male)	Rats (Female)	Rats (Male)	Rats (Female)
A	Control	43.43±1.33	49.48±5.73	5.99±0.33	6.38±0.41	8.16±0.21	8.26±0.13
B	T_{12}/N*	58.14±0.81^{a**}	59.86±1.91^{a}	3.91±0.41^{a**}	2.71±0.02^{a}	9.51±0.35^{a**}	9.58±0.13^{a}
C	T_{24}/N	55.08±2.55^{a}	62.58±0.1.00^{a}	3.32±0.31^{a}	2.16±0.04^{a}	9.72±0.36^{a}	9.79±0.24^{a}
D	T_{12}/P_{12}	45.29±3.79^{b,c}	44.69±7.24^{b,c}	5.04±0.07^{c}	5.55±0.11^{a,b,c}	9.00±0.10^{a,b,c}	9.04±0.15^{a,b,c}
E	T_{24}/P_{24}	47.88±3.06^{b,c}	50.98±6.36^{b,c}	5.81±1.12^{b,c}	4.60±0.54^{a,b,c,d}	9.10±0.36^{a,c}	9.02±0.06^{a,b,c}
F	P_{12}/T_{12}	45.09±2.65^{b,c}	51.21±3.51^{b,c,d}	7.56±1.00$^{a,b.c.d.e}$	6.28±0.53^{b,c,d,e}	8.50±0.55^{b,c,e}	8.72±0.50^{a,b,c}
G	P_{24}	40.97±0.59^{b,c}	43.57±2.36^{a,b,c,d,e,f}	6.74±1.07^{b,c,d}	6.67±0.21^{b,c,d,e}	8.06±0.22^{b,c,d,e}	8.14±0.06^{b,c,d,e,f}

*Control = Normal rat chow (N) and water with weekly subcutaneous injection of EDTA-saline solution.

T_{12}/N = Maintained on normal chow (N) while treating with 1, 2- dimethylhydrazine (DMH) for 12 weeks.

T_{24}/N = Maintained on normal chow but treated with 1, 2-dimethylhydrazine (DMH) for 24 weeks.

T_{12}/P_{12} = Treated with DMH for 12 weeks while on normal rat chow and later maintained on peanut diet (P) for12 weeks.

T_{24}/P_{24} = Treated with DMH and peanut simultaneously for12 weeks.

P_{12}/T_{12}= Maintained on peanut diet for 12 weeks, followed with DMH treatment for 12 weeks.

P_{24} = Maintained on peanut diet for 24weeks with weekly subcutaneous injection of EDTA- Saline solution.

**Values with superscripts a, b, c, d, e or f are significantly different from the value of the group with the corresponding upper case letter A, B, C, D, E or F ($p \leq 0.05$).

T represents DMH.

Discussion

Data from this study revealed a considerable reduction in total colon protein of rats in the groups exposed to the carcinogen relative to the control group in both sexes (Table 1). This protein loss agrees with earlier report by Okolie *et al*. (2013) that loss of tissue protein is usually one of the events in most cancers. The protein loss may have been due to increased rate of protein catabolism coupled with reduced synthesis (Evans *et al*., 2008; Tisdale, 2010). Proteins are also prone to ROS-induced damage and are a common target of amplified damage by free radicals (Perše, 2013) and toxic chemicals such as reactive methyldiazonium ion generated from DMH is not exempt from free radical production and attendant protein damage. Supplementation with peanut diet, however, restored the total protein level to normal. How peanut supplemented diet brought about this amelioration is not clear but if the free radical concept just mentioned is a factor then the antioxidant phytochemicals which we identified earlier (Isoje and Obi, 2021) and by others (Sanders *et a*l, 2000; Isanga and Zhang, 2007) must have mopped up the DMH associated free radicals and hence the tissue protein status restoration.

The results obtained from LDH activity assay in this study show that DMH exposure caused significant increase in colon activity of the enzyme. This finding agrees with earlier reports by Koukourakis *et al*. (2006) that lactate dehydrogenase (LDH) activities are often increased in cancer cells. This observation has been attributed to increased demand for energy which is readily obtained via anaerobic glycolysis sustained by lactate dehydrogenase activity. Okolie *et al*. (2013) report is also in consonance with our finding. They showed that the processes leading to colon cancer development significantly raised LDH activity in the colon of rats. Xu *et al*., (2005) reported that mitochondrial dysfunction associated with cancer results in the buildup of the glycolytic intermediate, pyruvate, hence increased activities of LDH through substrate-activation of LDH gene. Consumption of peanut diet had a lowering effect on LDH activities. This may be due to the high level of oil in peanuts which may have conditioned the cells to utilize the fatty acids of the oil rather than glucose for energy production (Allen *et al*., 2014). Additionally, lipid metabolism may have forced the cells to obtain their energy from mitochondrial metabolism, and it is generally believed that cancer cells have dysfunctional mitochondrial electron transport chain, thus resulting in up-regulation of one-electron reduction of O_2 causing ROS production. This phenomenon may cause only the cancer cells to experience oxidative stress while sparing the normal cells in such situations in which glucose metabolism is restricted (Allen *et al*., 2014).

Apart from cancer cells having enhanced anaerobic glycolytic process, they possess elevated pentose phosphate pathway activity (Aykin-Burns *et al.*, 2009). Glucose-6-phosphate dehydrogenase (G6PDH) is a critical enzyme in this pathway that produces reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate (R5P) from the pentose phosphate cycle. Data from this study showed that the activity of this enzyme was elevated in the colon of both sexes of rats that were carcinogen- treated relative to the control group rats (Table 2). Consumption of peanut supplemented diet caused a reduction in the activities of the enzyme (Allen *et al*., 2014). Tumour cells express a relatively high level of glucose-6-phosphate dehydrogenase due to their high demand for NADPH that promotes cancer cell proliferation, growth and survival (Patra and Hay, 2014). Consumption of peanut supplemented diet may have decreased the ability of DMH co-exposed cells to sustain high glucose-6- phosphate dehydrogenase activity and associated NADPH production due to limited glucose availability during peanut supplemented diet consumption. Evidence from research studies suggest that metabolism of fat in most tissues does not facilitate gluconeogenesis and hence low glucose-6-phosphate formation, low pentose phosphate pathway activities and ultimately heightened stress status in neoplastic cells compared to normal cells (Allen *et al*., 2014).

Conclusively, DMH associated glucose consumption and pattern of metabolism as in exposed rat colon is altered by the consumption of peanut supplemented diet. We believe that peanut phytochemical and oil content have fundamental roles to play in the altered glucose metabolism.

References

Allen BG, Bhatia SK, Anderson CM, Eichenberger-Gilmore JM, Sibenaller ZA, Mupuskar KA, Schoenefeld JD, Buatti JM, Spitiz DR Fath MA: Ketogenic diets as an adjuvant cancer therapy: History and potential mechanism. Redox Biol, 2**:** 963-970. 2014.

Animal Care and Use Program. In, *Guidelines for Care and Use of Animals for Scientific Research*, 8th ed, Washington: National Academic Press. 11-13. 2011.

Aykin-Burns N, Zhu AZ, Oberley LW, Spitz DR: Increased levels of superoxide and H_2O_2 mediates the differential susceptibility of cancer cells versus normal cells to glucose deprivation. Biochem J, 418(1): 29-37. 2009.

Deutsche Gesellschaft für Klinische Chemie (DGKC): Determination of Lactate Dehydrogenase Activity Using UV Method. J Clin Chem Clin Biochem, 8: 658. 1970.

Evans WJ, Morly JE, Agriles J, Bales C, Baracos V, Guttridge D, Jatoi A, Kalantor ZK, Lochs H: Cachexia: A new definition. Clin Nutri, 27: 793-799. 2008.

Guyton M, Verghese M, Walker LT, Chawan CB, Shackelfold L, Jones J, Kwatiwada J: Inhibotory effects of feeding selected levels of peanuts on axoxymethane-induced aberrant crypt foci in male fisher 344 rats. Int J Cancer Res, 4(4): 146-143. 2008.

Hagland HR, Søreide K: Cellular metabolism in colorectal carcinogenesis: Influence of lifestyle, gut microbiome and metabolic pathways. Cancer Lett, 356(2 Pt A): 273-80. 2015. doi: 10.1016/j.canlet.2014.02.026. Epub 2014 Mar 12. PMID: 24614287.

Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. Cell, 144(5): 646-674. 2011

Isanga J, Zhang G: Biologically active components and neutraceuticals in peanuts and related products: Review. Food Rev Int, 23:123-140. 2007.

Isoje AO: Evaluation of the Anti-carcinogenic Effect of *Arachis hypogaea* Seeds on 1, 2 –Dimethyhydrazine-Induced Carcinogenesis in Rat Colon. PhD Thesis, University of Benin. 2021.

Isoje AO, Obi FO: Nutritional and phytochemical components and *in vitro* antioxidant capacity of raw *Arachis hypogaea* seeds. J Med Plants Stud, 9(2): 35 - 39. 2021.

Koukourakis MI, Giatromanolaki A, Sivridis E, Gatter KC, Harris AL: Lactate dehydrogenase 5 expression in operable colorectal cancer: strong association with survival and activated vascular endothelial growth factor pathway–a report of the Tumour Angiogenesis Research Group. J Clin Oncol, 24:4301- 4308. 2006.

Lohr GW, Waller HD: Glucose-6-Phosphate Dehydrogenase. Methods of Enzymatic Analysis, 3rd edition, Varlag Chemie, Wehnheim, pp. 636-643, 1974.

Moreno-Sanchez S, Rodriguez-Enriquez A, Marin-Hernandez E, Saavedra S: Energy metabolism in tumor cells. FEBS J, 274: 1393–1418. 2007.

Okolie NP, Agu K, Eze, GI: Proctective effect of ethanolic leaf extract of *Annona muricata* linn on some early events in Cycas-induced colorectal carcinogenesis in rats. J Pharm Sci Innov, 2(4): 14-21. 2013.

Patra KC, Hay N: The pentose phosphate pathway and cancer. Trends Biochem Sci, 39(8): 347-354. 2014.

Perše M: Oxidative Stress in the Pathogenesis of Colorectal Cancer: Cause or Consequence? Biomed Res Int, 2013: 725710. 2013.

Sanders TH, McMichael RW, Hendrix KW: Occurrence of resveratrol in edible peanuts. J Agric Food Chem, 48(4): 1243-1246. 2000.

Tisadale MJ: Reversing Cachexia. Cell, 142(4)**:**511-512. 2010.

Večerić Z, Cerar A: Comparison of Wistar vs. Fischer rat in the incidence of 1, 2-dimethylhydrazine induced intestinal tumors. Radiol Oncol, 38(3): 227-234. 2004.

Ward PS, Thompson CB: Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. Cancer Cell, 21(3): 297–308. 2012.

Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P: Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res, 65(2): 613-621. 2005.