MUTATION ANALYSIS OF K-*RAS* GENE EXON 1 AND 2 IN PRIMARY RAT LIVER CELLS CULTURE INDUCED BY *N*-METHYL-*N*-NITROSOUREA: INHIBITION EFFECTS OF EGCG FROM GREEN TEA

Djoko Agus Purwanto

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Airlangga University, Indonesia.
E-mail: djokoagus@yahoo.com

Abstract

Cancer is remains a major health problem world-wide. Cancer control strategy such as chemoprevention needs to be developed to reduce cancer incident. In this case, the consumption of green tea (*Camellia sinensis*) beverage apparently plays an important role. One potential considerable mechanism is the strong antioxidant and anti-mutation effect of green tea major component, (-)-epigallocatechin gallate (EGCG). Objective of this research is develop an analysis polymerase chain reaction-single strain conformational polymorphism (PCR-SSCP) technique to determine the inhibition effect of EGCG on K-*ras* genes exon 1 and 2 mutation induced by N-methyl-N-nitrosourea (MNU). Effect of EGCG 8.3, 16.6, 33.3 and 66.7 ppm on K-*ras* exon 1 and exon 2 mutation were analyzed. The mutation was induced by *N*-methyl-*N*-nitrosourea (MNU) 8, 16, 32 and 48 μM and determined using a PCR-SSCP technique in primary Wistar rat liver cells culture. Mutation in K-*ras* genes were confirmed also by DNA Sequencing method. K-*ras* exon 1 codon 12 mutation was detected in all of control and 8.3 ppm of EGCG exposure. EGCG 16,6 ppm and higher showed that strongly inhibition of K-*ras* exon 1 mutation except 48 μM MNU, while exon 2 didn't show any mutation in codon 61. DNA sequencing analysis in codon 12 showed single point mutation from GGT (glycine) to GCT (alanine). Conclusion**,** PCR-SSCP technique can be used to determine

K-ras genes mutation and these findings suggest that EGCG from green tea significantly reduces the incidence of K-*ras* exon 1 mutation induced by MNU therefore has strong potential as a useful therapeutic regimen for inhibiting cancer development.

Keywords: PCR-SSCP, mutation K-*ras* exon 1, K-*ras* exon 2, MNU, EGCG, green tea, cancer chemoprevention

Introduction

Tea is one of the most popular beverages consumed worldwide. Tea drinking has been associated with decreased occurrence of cancer and heart disease. One potential mechanism for these findings is the strong antioxidant effect of tea polyphenols. Tea, from the plant *Camellia sinensis*, is consumed in different parts of the world as green, black, or oolong tea. Tea contains many compounds, especially polyphenols, and epidemiological studies show that polyphenolic compounds present in tea reduce the risk of a variety of diseases (Jian et al., 2004). Green tea polyphenols have demonstrated to be an effective chemopreventive agent (Cabrera et al., 2003; Gossslau and Chen, 2004). Moreover, some epidemiological studies have associated the consumption of tea with a lower risk of several types of cancer (Sato and Myata, 2000; Toit at al., 2001). There is good evidence from in vitro studies that green tea catechins have an important role in protection against degenerative diseases. (-)-Epigalocatechin gallate (EGCG) from green tea extract has many activities such as anticarcinogenic (Beltz et al., 2006; Spinella et al., 2006), antioxidant (Luximon-Ramma et al., 2006), anti microbial activities (Paul et al., 2006; Watson et al., 2006) as well as anti diabetic activity (Ts rat m uneki et al., 2004).

In this research, the effect of EGCG have been studying on K-*ras* mutation induced by direct application of *N*-methyl-*N*-nitrosourea (MNU**)**. Activation of the *ras* genes by point mutation has been observed in many tumors and cancer, including tumors induced by MNU. Because of that reasons, the method of mutation analysis is very important to be developed. Usually, K-*ras* protooncogene is altered by point mutations on codon 12, 13, or 61 in a wide variety of tumors (Fearon, 1993). The detection of K-*ras* mutations enables understanding of cancer biology and pathogenesis. Moreover, alterations involving this oncogene may be of clinical importance because they can provide information for early diagnosis and prognosis (Puig etal., 2000; Schimanski et al., 1999; Cerottini et al., 1998). Recently, we developed PCR-SSCP method to detect K-*ras* gene mutations in primary rat liver cell culture induced by MNU and inhibited by EGCG from green tea. The results of amplified DNA were analyzed by DNA sequencer to confirm type of mutation in K-*ras* exon 1 and 2.

Material and Method

Material and instrument

EGCG and *N*-methyl-*N*-nitrosourea were purchased from Sigma Chemical (St. Louis, MO). Wistar rat was obtained from Animal Laboratory, Faculty of Pharmacy, Airlangga University and Thermoline CS-1 thermocycler was used for DNA amplification. For DNA sequencing, it has been used DNA sequencer ABI-Prism Model 310 version 3.0.

Isolation and culture of rat hepatocytes.

Wistar rats were anesthetized with Chloroform. Hepatocytes were isolated by perfusion and disintegration solution (Binda et al., 2003). Cell viability, estimated by Trypan blue exclusion, was >80%. Isolated hepatocytes were resuspended in +WME supplemented with FCS (10%), insulin (4 mg/L), hydrocortisone (10^{-5} mol/L) and gentamicin (50 mg/L). Cell incubations were performed at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 4 h, the medium was renewed to remove unattached hepatocytes and after 12–14 h, cells were shifted to a serum-free medium, consisted of -WME supplemented with glutamine (2 mmol/L), insulin (4 mg/L), hydrocortisone (10^{-5} mol/L) and gentamicin (50 mg/L). At this time point, cultures were exposed to various concentrations of MNU and EGCG. Subsequently, the medium was changed daily.

DNA isolation

DNA from rat liver cell culture was prepared by proteinase K digestion and phenol-chloroform extraction as described previously (Sambrook et al., 1989).

DNA Amplification

Exons 1 and 2 of the K-ras gene were amplified individually by PCR using certain primer sequences below.

Exon 1

Sense : 5'-TTTTTATTATAAGGCCTGCT-3'

Antisense : 5'-GTCCACAAAATGATTCTGAA-3'

Exon 2

Sense : 5'-ACCTGTCTCTTGGATATTCT-3'

Antisense : 5'-TGATTTACTATTATTTATGG-3'

The PCR was carried out in a total volume of 50 µL containing the following: 250 ng of extracted DNA, 200 µM dNTP, 2 U of Taq DNA polymerase (Perkin-Elmer), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1 g/L gelatin. The PCR was performed with 32 cycles (95 °C for 1 min, 48 °C for 45 s, and 72 °C for 1.5 min). The PCR was performed with 30 cycles and began with hot start 95^{o}C 5 min and 85^{o}C 1 min and extra-extension 72^{o}C 10 min. The PCR was carried out with 12 pmol of each PCR primer (K-*ras* exon 1 and 2). Experimental conditions to avoid PCR product carryover were applied according to previous research (Kwok and Higuchi, 1989).

SSCP analysis

The amplified product was denatured at 94°C for 5 min along with 4X formamide-BPB dye and was snap chilled on ice. The product was subjected to electrophoresis on 0.5 x MDE, (mutation detection enhancer) gel (BMA products, USA) with 10% glycerin on Hoefer SQ 3 sequencer at 500 V for 8 hr. The gels were silver stained, vacuum dried and analysed for SSCP band patterns.

Result and Discussion

Amplification of K-ras gene exon 1 and exon 2 yielded 115 bp and 117 bp fragment length of DNA respectively (fig.1). Some PCR conditions were tried to get optimum results. Also some PCR program have done to get one band of DNA fragment. Optimum condition is 30 cycles with 95^{o}C 1 min, 48^{o}C 45 s, 72^{o}C 1,5 min. The PCR

program was began with hot start 95^{o}C 5 min and 85^{o}C 1 min and last program was extra-extension 72^{o}C for 10 min. Figure 1 shows that only one band in agarose gel, the band below of PCR product is excess primer that can discharge by electro elution technique.

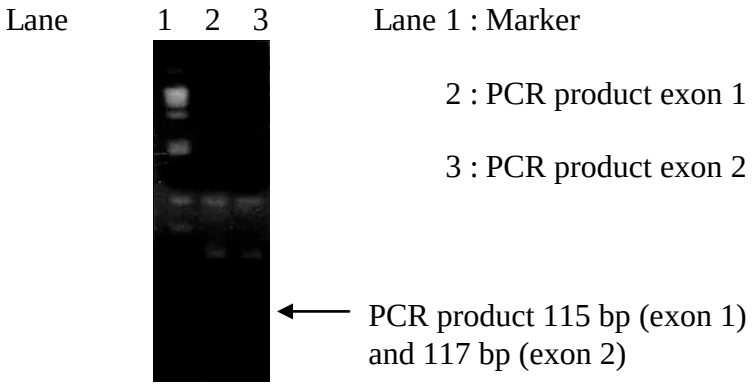

Figure 1. Amplification of K-*ras* gene exon 1 and exon 2 in rat hepatocytes DNA using Thermoline CS-1 thermocycler with program 95^{o}C 1 min, 48^{o}C 45 s, 72^{o}C 1,5 min. The PCR was performed with 30 cycles and began with hot start 95^{o}C 5 min and 85^{o}C 1 min and extra-extension 72^{o}C 10 min.

PCR SSCP assay of K-*ras* gene exon 1 shows some mutation when treated by MNU, while exon 2 there are no mutation can be detected. All of control and EGCG 8.3 ppm exposure to MNU- induced mutation cell culture, underwent mutation in K-*ras* exon 1 (fig. 2)\, while EGCG 16.6 ppm (except 48 µM MNU),and higher concentration showed that strongly inhibition of K-*ras* exon 1 mutation (Table 1.). This fact proved that

EGCG in low concentration (below 16.6 ppm) can not prevent mutation in K-*ras* gene induced by MNU, but in higher concentration EGCG is effective substance for prevent mutation. Extra band in polyacrylamide gel from PCR-SSCP proves mutation in K-*ras* gene.

Figure 2. PCR-SSCP product of K-*ras* exon 1 on some of hepatocytes cell culture treated by EGCG and MNU using gel polyacrylamide 8% and running for 3 h 150 volt , 10^{o}C. Lane (1) WME media (control) (2) MNU 48 µM; (3) MNU 32 µM; (4) MNU 16 µM; (5) MNU 8 µM; (6) MNU 8 µM + EGCG 8.3 ppm; (7) MNU 16 µM + EGCG 8.3 ppm; (8) MNU 32 µM + EGCG 8.3 ppm; (9) MNU 48 µM + EGCG 8.3 ppm; (10) MNU 48 µM + EGCG 16.6 ppm;

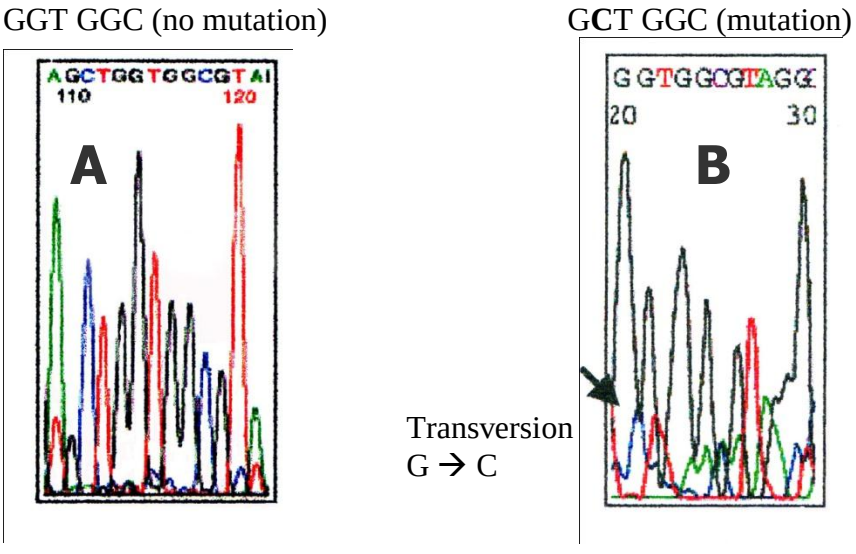

Figure 3. Sequence analysis of amplified product of K-*ras* gene exon 1 codon 12+13 using DNA *sequencer* ABI-Prism Model 310. A) treated with MNU 8 µM and EGCG 16,6 ppm, no mutation; B) treated with MNU 8 µM only, mutation G to C (transversion).

Figure 3 shows that treat with MNU 8 µM only cause mutation in codon 12 from G to C. Adding of EGCG in hepatocytes cell culture prevent nitronium ion attack to DNA and mutation will not occur. Antioxidant and free radical scavenger mechanism may be suspected to explain this phenomena.

Tabel 1. Effect of EGCG on K-*ras* exon 1 mutation in rat hepatocyte DNA induced by MNU using PCR-SSCP method on polyacrylamide gel 8% and running for 3 hours 150 volt , 10^{o}C (*n*=3)

Treatment on hepatocyte cell culture	Mutation on proto-oncogen K-*ras* exon 1	
	Control	EGCG

			8,3 ppm	16,6 ppm	33,3 ppm	66,7 ppm
Control		-	-	-	-	-
+ MNU	8 µM	+	+	-	-	-
	16 µM	+	+	-	-	-
	32 µM	+	+	-	-	-
	48 µM	+	+	+	-	-

(+) = mutation ; (-) = no mutation

In summary, it was concluded that MNU exposure to liver rat cell culture affect mutation in K-ras exon 1 but not at exon 2. Because of that reason, the effect of EGCG on mutation inhibition induced by MNU can not be investigated in exon 2. EGCG 8.3 ppm can not prevent MNU attack in DNA caused mutation, begin 16.6 ppm until 66.7 ppm EGCG can prevent mutation induced by MNU. These results suggest that EGCG from green tea significantly has strong potential as a useful therapeutic regimen for inhibiting cancer development.

Acknowledgement

This study was supported by RUT grant from Indonesia Government. We would like to thank Prof.Dr.Noor Cholies Zaini, Apt. and Prof. Sofia Mubarika H.,dr., M.Med.Sc., Ph.D. for their supervising in this research.

Refference

Beltz, L.A., Bayer, D.K., Moss, A.L., and Simet, I.M., 2006. Mechanisms of cancer prevention by green tea and black tea polyphenols. Anticancer Agent Med. Chem., 6(5):389-406.

Binda, D., Lasserre-Bigot, D., Bonet, A., Thomassin, M., Come, M. P., Guinchard, C., Bars, R., Jacqueson, A. & Richert, L., 2003. Time course of cytochromes P450 decline during rat hepatocyte isolation and culture: effect of L-NAME. Toxicol. In Vitro. 17:59-67.

Cabrera, C., Gimenez, R., and López, M.C. 2003. Determination of tea components with antioxidant activity. J. Agric. Food Chem., (51):4427-35.

Cerottini, J.P., Caplin, S., Saraga, E., Givel, J.C., Benhattar, J., 1998. The type of K-ras mutation determines prognosis in colorectal cancer. Am J Surg;175:198-202.

Fearon, E.R, 1993. K-ras gene mutation as a pathogenetic and diagnostic marker in human cancer. J Natl Cancer Inst.;15:1978-1980.

Gosslau, A., and Chen, Y., 2004. Nutraceuticals, Apoptosis, and Disease Prevention, Nutrition, (20): 95-101.

Jian, L., Xie, L. P., Lee, A. H. & Binns, C. W., 2004. Protective effect of green tea against prostate cancer: a case-control study in southeast China. Int. J. Cancer 108:130-135.

Kwok, S., and Higuchi, R., 1989. Avoiding false positives with PCR. Nature;339:237-238.

Luximon-Ramma, A., Neergheen, V.S., Bahorun, T., Crozier, A., Zbarsky, V., Datla, K.P., Dexter, D.T., Aruoma, O.I., 2006. Assessment of the phenolic

composition of the organic extracs of Mauritian black teas: A potential contributor to their antioxidant functions. Biofactors., 27(1-4):79-91.

Paul, D.S., Saroj, S., Yukihiko, H., and Peter, W.T., 2006. Potentiation of catechin gallate-mediated sensitization of Staphylococcus aureus to oxacillin by nongalloylated catechin. Antimicrob. Agents Chemother., 50(2):752-755.

Puig P, Urgell E, Capella G, Sancho FJ, Pujol J, Boadas J, 2000. A highly sensitive method for K-ras mutation detection is useful in diagnosis of gastrointestinal cancer. Int J Cancer, 85:73-77.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual. New York. Cold Spring Harbor Laboratory Press, p. 9-16.

Sato,T.; Myata, G., 2000. The Nutraceutical Beneft, Part I: Green tea. Nutrition, v. 16, p. 315-317.

Schimanski CC, Linnemann U, Berger M.R., 1999. Sensitive detection of K-ras mutations augments diagnosis of colorectal cancer metastases in the liver. Cancer Res;59:5169-5175.

Spinella, F., Rosano, L., DiCastro, V., Decandia, S., Albini, A., Nicotra, M.R., Natali, P.G., and Bagnato, a., 2006. Green tea polyphenol epigallocatecin 3-gallate inhibits the endothelin axis and downstream signaling pathways in ovarian carcinoma. Mol. Cancer Ther., 5 (6):1483-92.

Toit, R.; Volsteedt, Y.; Apostolides, Z., 2001. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology, v. 166, p. 63-69,

Tsuneki, H., Ishizuka, M., Terasawa, M., Wu, J., Sasaoka, T., and Kimura, 2004. Effect of green tea on blood glucose levels and serum proteomic patterns in diabetic (db/db) mice and on glucose metabolism in healthy humans. BMC Pharmacol., 4:18.

Tsuneki, H., Ishizuka, M., Terasawa, M., Wu, J., Sasaoka, T., and Kimura, 2004. Effect of green tea on blood glucose levels and serum proteomic patterns in diabetic (db/db) mice and on glucose metabolism in healthy humans. BMC Pharmacol., 4:18.

Watson, J.L., Vicario, M., Wang, A., Moreto, M., McKay, D.M., 2005. Immune cell activation and subsequent epithelial dysfunction by Staphylococcus enterotoxin B is attenuate by green tea polyphenol (-)-epigallocatechin gallate. Cell Immunol., 237(1):7-16.

Watson, J.L., Vicario, M., Wang, A., Moreto, M., McKay, D.M., 2005. Immune cell activation and subsequent epithelial dysfunction by Staphylococcus enterotoxin B is attenuate by green tea polyphenol (-)-epigallocatechin gallate. Cell Immunol., 237(1):7-16.