PKCα Suppresses 7,12-Dimethylbenz[*a*]anthracene-induced Skin Tumor Formation

TAKESHI HARA, SHOJI MATSUMURA, FUMIHIKO HAKUNO, SHIN-ICHIRO TAKAHASHI and KAZUHIRO CHIDA

Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Abstract. *Background: Protein kinase C (PKC)α is distributed in almost all tissues and participates in various signaling pathways. However, the role of PKCα in carcinogenesis remains unclear. In this study, we performed complete skin carcinogenesis in PKCα knockout mice by repeated administration of 7,12- dimethylbenz[a]anthracene (DMBA). Materials and Methods: Complete skin carcinogenesis was performed by repeated DMBA treatment using PKCα knockout mice. The number of tumors was determined weekly. Tumor types were determined by Hematoxylin and eosin (H & E) analysis. Tumor growth was assayed by proliferating cell nuclear antigen (PCNA) staining. Results: In the knockout mice, the average number of tumors was 16.6/mouse at 20 weeks. In contrast, in the wild-type (WT) mice, the tumor number was 6.9/mouse. Growth and malignant grade of tumors in PKCα knockout mice did not differ from those in WT mice. Conclusion: PKCα suppresses tumor formation, but not tumor growth and progression in skin carcinogenesis.*

Protein kinase C (PKC) belongs to the phospholipid-dependent serine/threonine kinases that are activated by membrane lipids produced by various extracellular stimuli (1). Based on their structural similarities and co-factor dependence, there are 10 isotypes in three subfamilies. The conventional PKCs (cPKCα, βI, βII, and γ), the novel PKCs (nPKCδ, ε, η, and θ), and the atypical PKCs (aPKCζ and λ/ι). cPKC and nPKC isotypes are the major intracellular targets for potent mouse tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), suggesting that these PKC isotypes have an important role in tumor formation (1, 2).

Correspondence to: Kazuhiro Chida, Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan. Tel/Fax: +81 358418152, e-mail: acchida@mail.ecc.u-tokyo.ac.jp

Key Words: DMBA, knockout mouse, PKCα, skin carcinogenesis, tumor progression.

Of the PKC isotypes, PKCα is distributed in almost all tissues and participates in various signaling pathways including cell-cycle regulation, differentiation, and apoptosis (3). Several lines of evidence suggest that PKCα is involved in carcinogenesis. Previously, we performed a two-stage skin carcinogenesis study in which 7,12-dimethylbenz[*a*]anthracene (DMBA) was used as an initiator and TPA as a promoter in *PKCα* knockout mice, which exhibited increased susceptibility to tumor formation, however, no squamous cell carcinoma (SCC) was observed in the study (4). *PKCα* knockout mice exhibit increased susceptibility to intestinal tumor formation in $Apc^{Min/+}$ mice (5). These reports suggest that PKCα functions as a tumor suppressor. In contrast, PKCα promotes tumor growth and progression in certain tumors and cell lines (6-8). Using the DMBA/TPA protocol, PKCα overexpression in the epidermis was found to induce in an more dimplified manner skin tumor formation, compared to control mice (9). However, other studies found that PKCα overexpression in the epidermis did not affect skin tumor formation using the DMBA/TPA protocol (10, 11). Therefore, the role of PKCα in carcinogenesis is controversial. Other approaches may be necessary to elucidate PKCα function in tumor formation. In this study, in order to obtain further insight into the function of PKCα in tumor formation, we performed complete skin carcinogenesis by repeated DMBA treatment in *PKCα* knockout mice.

Materials and Methods

Mice. PKCα knockout mice with a genetic background of C57BL/6J and 129/SvJ were used as reported previously (4). All experiments were performed in accordance with the policies of the Animal Ethics Committee of The University of Tokyo.

Carcinogenesis. Eight-week-old female, wilde type (WT) and *PKCα* knockout mice were used. The dorsal skin was shaved and one week later, DMBA (100 μg; Sigma, St Louis, MO, USA) dissolved in 0.2 ml acetone was applied to the shaved area, once weekly for 20 weeks. The number of tumors was determined weekly.

Histological analysis. The mice were killed by cervical dislocation and their dorsal skin or tumor samples were removed, and fixed

Table I. *Histological analysis of skin tumors from wild-type (WT) and knockout (KO) mice at 20 weeks after repeated treatment with 7,12-dimethylbenz[a]anthracene (DMBA). All of the mice used survived for the duration of the carcinogenesis experiments. The difference in malignancy grade between WT and KO mice was not statistically significant using the χ^2 test (p>0.1).*

	WT		KO	
Group	n	%	n	%
A: Benign tumor	11	25	19	27
B: Severely dysplastic papilloma with carcinoma *in situ* and/or focal invasion	11	25	12	17
C: Well-differentiated SCC	15	34	29	41
D: Moderately-differentiated SCC	6	14	6	9
E: Poorly-differentiated SCC or spindle cell carcinoma	1	2	4	6

All the mice employed in the the study survived for the duration of the carcinogenesis experiments. The difference in malignancy grade between WT and KO mice was not statistically significant using the χ^2 test (p>0.1).

in 4% formaldehyde in phosphate-buffered saline at room temperature and embedded in paraffin. Skin and tumor sections (4-μm-thick) were deparaffinized with xylene and ethanol, and stained with H&E. Tumor types were determined by H&E analysis according to criteria previously described (12, 13). The tumors were subdivided into five groups (Table I, A-E). Tumors with well-differentiated hyperplastic lesions without marked atypia of all layers of the epidermis, and with intact basement membranes, were classified as group A. Tumors with lesions with marked atypia and mitotic figures in all layers of the epidermis, with lesions equivalent to carcinoma *in situ*, or with focal invasions were classified as group B. SCCs were characterized by endophytic growth that progressively invaded the dermis and the subcutaneous tissue. SCCs with marked horny cells (terminally differentiated cells) were classified as group C. SCCs with differentiated cells but no clear horny pearls were classified as group D. SCCs with small areas of squamous differentiation, including spindle cell carcinoma, were classified as group E.

Proliferating cell nuclear antigen (PCNA) staining. Deparaffinized tumor sections were subjected to antigen retrieval with 10 mM citrate buffer (pH 6.0) at 95˚C for 20 min, followed by treatment with 3% hydrogen peroxide for 10 min at room temperature. Sections were blocked with 5% normal goat serum and incubated with an anti-PCNA antibody conjugated with peroxidase (Dako Japan, Kyoto, Japan) at 37˚C for 1 h. Diaminobenzidine was used as a chromogen. Hematoxylin was used for counterstaining. The number of PCNA-positive cells was determined from at least four independent fields.

Statistical analysis. Statistical analysis of the experiments was performed using the two-tailed unpaired Student's t-test, except for histological analysis of skin tumors, which was performed using the χ^2 test. *p*-Values <0.05 were accepted as demonstrating significant differences between groups.

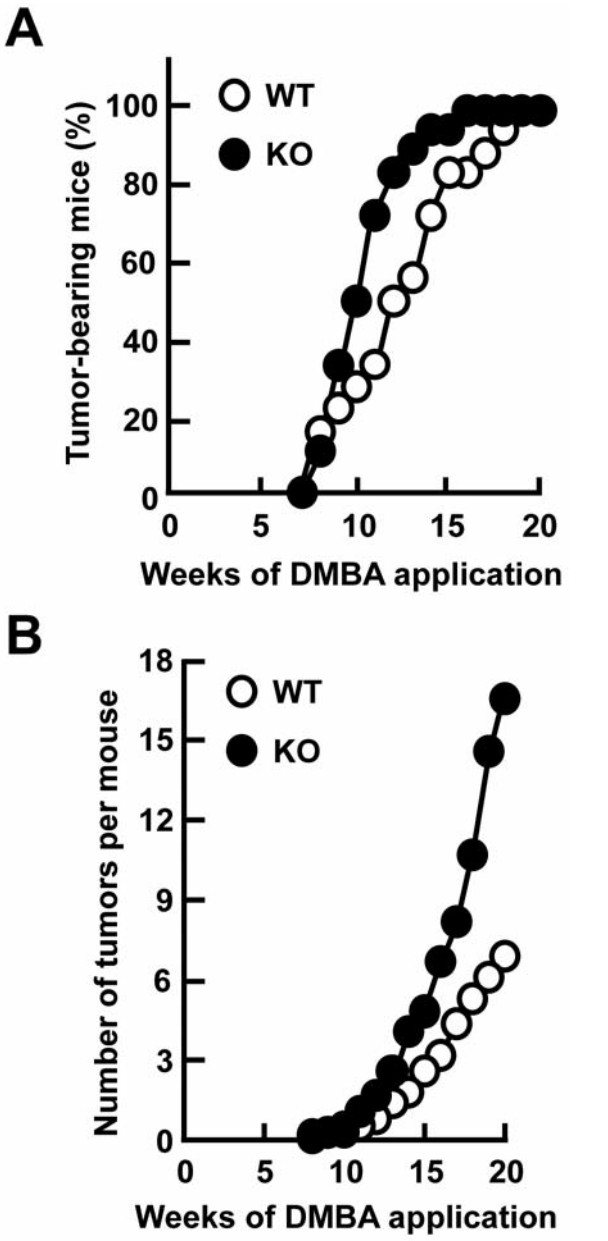

Figure 1. *Time-dependent changes of tumor formation in complete skin carcinogenesis. Data of the wild-type (WT) mice and knockout (KO) mice are shown. WT and KO mice were treated repeatedly with DMBA, once weekly, for 20 weeks. Eighteen mice were used for each group. A: Incidence of tumors (percentage of tumor bearers). B: Average number of tumors per mouse. KO mice were significantly different from WT mice in the number of tumors at weeks 11 (p<0.05), 16 (p<0.01), and 20 (p<0.01).*

Results

We performed complete skin carcinogenesis by repeated DMBA treatment. The first tumors appeared after eight weeks of DMBA treatment in *PKCα* knockout and WT mice. The knockout mice exhibited rapid tumor development

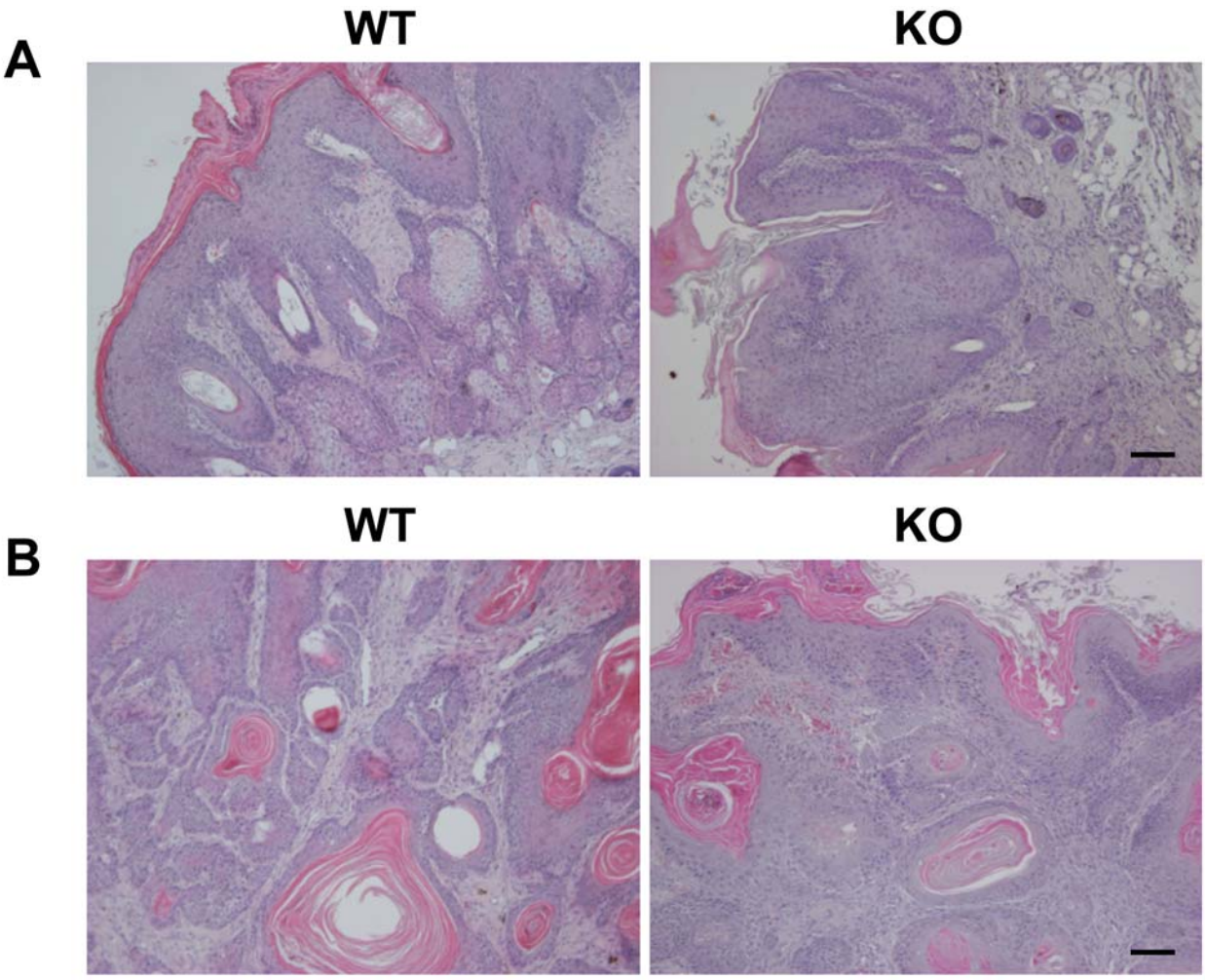

Figure 2. *Hematoxylin and eosin (H&E) staining of tumor sections from the wild-type (WT) and knockout (KO) mice. Representative figures are shown. A: Benign tumor. B: Well-differentiated squamous cell carcinoma. Scale bar=100 μm.*

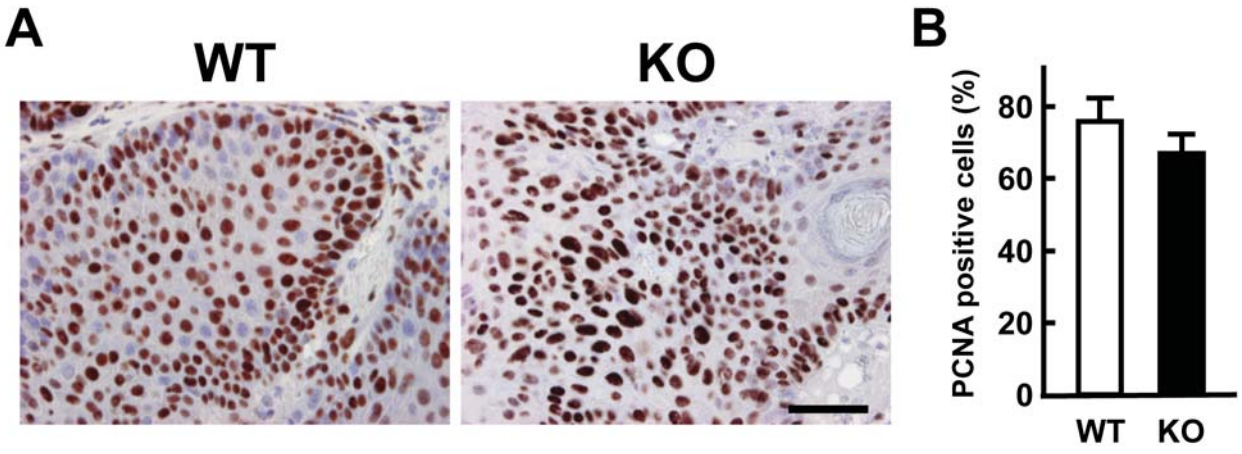

Figure 3. *A: Proliferating cell nuclear antigen (PCNA) staining in a representative section of skin tumors. Scale bar=30 μm. Counterstaining was performed with hematoxylin. B: Percentage of PCNA-positive cells in tumors from the wild-type (WT) and knockout (KO) mice including benign tumors and squamous cell carcinomas (n=6). There was no significant difference between WT and KO mice (p>0.1). Results are shown as mean±SE.*

compared with WT mice (Figure 1A and B). At 11 weeks, the proportion of tumor-bearing mice reached 72% in the KO mice, whereas it only reached 33% in the WT mice. The number of tumors in the knockout mice was 1.1±0.2/mouse (mean±SE). In contrast, the number of tumors in the WT mice was 0.4±0.2/mouse ($p<0.05$). At 20 weeks, all knockout and WT mice had developed tumors. The knockout mice developed 16.6±2.7 tumors/mouse. In contrast, in the WT mice, there were only 6.9±0.8 tumors/mouse ($p<0.01$). Repeated application of acetone as a solvent control induced no tumors. These data indicate that the knockout mice were significantly more susceptible to skin tumor formation than the WT mice regarding skin carcinogenesis.

We evaluated tumors ≥2 mm in diameter from knockout and WT mice for histopathological features at the end of the experiment (Figure 2). In the knockout mice, 56% of tumors were SCC, and 14% were moderately or poorly differentiated SCC. In the WT mice, 50% of tumors were SCC, and 16% were moderately or poorly differentiated (Table I). There were no significant differences in the malignancy grade between knockout and WT mice (Table I, $p>0.1$, χ^2 test), which indicates that PKCα was not essential for malignant progression in skin carcinogenesis.

To determine whether PKCα regulates tumor growth or not, we stained tumor sections with an antibody against PCNA, which is a proliferation marker (Figure 3). The percentage of PCNA-positive cells in the knockout mice was 63±6.1% (mean±SE), and it was 65±3.8% in WT mice. No significant difference in the percentage of PCNA-positive cells was observed in tumors between knockout and WT mice, which is consistent with our previous study (4). These data indicate that PKCα was not essential for tumor growth in skin carcinogenesis.

Discussion

In this study, the *PKCα* knockout mice were significantly more susceptible to DMBA-induced skin tumor formation. These results show that PKCα negatively regulates tumor formation, which is consistent with our previous work and other studies (4, 5, 14, 15). Our findings provide additional evidence that PKCα suppresses tumor formation. The malignant grade of tumors and tumor growth in *PKCα* knockout mice were no different from those in WT mice. These results indicate that PKCα is not essential for malignant progression and tumor growth. It is important to investigate the mechanisms that underlie carcinogen-induced tumor or carcinoma formation. DMBA is a polycyclic aromatic hydrocarbon and an abundant environmental contaminant (16). Thus, *PKCα* knockout mice could be a useful model for elucidating the underlying mechanisms that are involved in the response to environmental carcinogens linked to the development of human SCC.

The mouse skin carcinogenesis model is useful for understanding the multi-stage nature of human carcinogenesis (17). Previous studies of our group suggested that PKCα suppresses skin tumor promotion (4). It is likely that PKCα inhibits selective clonal expansion of initiated cells under the effects of TPA (17). Presently our findings suggest that PKCα may have a protective role during the initiation stage in skin tumor formation. Therefore, we propose that PKCα suppresses both tumor initiation and tumor promotion in skin tumor formation.

Based on our findings, it is possible that PKCα suppresses tumor formation, but not tumor growth and tumor progression in human. Thus, inhibition of PKCα may not be useful for cancer therapy. In fact, PKCα-specific antisense oligonucleotide, displayed no or only slight effect on advanced non-small cell lung carcinoma patients or on metastatic colorectal cancer in patients (18, 19). Additional approaches are necessary for the development of specific PKCα inhibitors or activators, which will act as anticancer drugs.

Acknowledgements

The Authors thank Hideyuki Ozawa and Maho Shida (Graduate School of Agricultural and Life Sciences, The University of Tokyo) for their technical assistance. This research was supported by a Grant-in Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Ohno S and Nishizuka Y: Protein kinase C isotypes and their specific functions: prologue. J Biochem *132*: 509-511, 2002.
2 Mueller MM: Inflammation in epithelial skin tumours: Old stories and new ideas. Eur J Cancer *42*: 735-744, 2006.
3 Nakashima S: Protein kinase C α (PKC α): regulation and biological function. J Biochem *132*: 669-675, 2002.
4 Hara T, Saito Y, Hirai T, Nakamura K, Nakao K, Katsuki M and Chida K: Deficiency of protein kinase Cα in mice results in impairment of epidermal hyperplasia and enhancement of tumor formation in two-stage skin carcinogenesis. Cancer Res *65*: 7356-7362, 2005.
5 Oster H and Leitges M: Protein kinase C α but not PKCζ suppresses intestinal tumor formation in $Apc^{Min/+}$ mice. Cancer Res *66*: 6955-6963, 2006.
6 Dean N, McKay R, Miraglia L, Howard R, Cooper S, Giddings J, Nicklin P, Meister L, Ziel R, Geiger T, Muller M and Fabbro D: Inhibition of growth of human tumor cell lines in nude mice by an antisense of oligonucleotide inhibitor of protein kinase C-α expression. Cancer Res *56*: 3499-3507, 1996.
7 Haughian JM, Reno EM, Thorne AM and Bradford AP: Protein kinase C alpha-dependent signaling mediates endometrial cancer cell growth and tumorigenesis. Int J Cancer *125*: 2556-2564, 2009.
8 Tan M, Li P, Sun M, Yin G and Yu D: Up-regulation and activation of PKCα by ErbB2 through Src promotes breast cancer cell invasion that can be blocked by combined treatment with PKCα and Src inhibitors. Oncogene *25*: 3286-3295, 2006.

9 Cataisson C, Ohman R, Patel G, Pearson A, Tsien M, Jay S, Wright L, Hennings H and Yuspa SH: Inducible cutaneous inflammation reveals a protumorigenic role for keratinocyte CXCR2 in skin carcinogenesis. Cancer Res *69*: 319-328, 2009.
10 Wang HQ and Smart RC: Overexpression of protein kinase C-α in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF-α expression but not tumor promotion. J Cell Sci *112*: 3497-3506, 1999.
11 Jansen AP, Dreckschmidt NE, Verwiebe EG, Wheeler DL, Oberley TD and Verma AK: Relation of the induction of epidermal ornithine decarboxylase and hyperplasia to the different skin tumor-promotion susceptibilities of protein kinase Cα, -δ and -ε transgenic mice. Int J Cancer *93*: 635-643, 2001.
12 Aldaz CM, Conti CJ, Klein-Szanto AJ and Slaga TJ: Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: relevance to malignancy. Proc Natl Acad Sci USA *84*: 2029-2032, 1987.
13 Klein-Szanto AJP: Pathology of human experimental skin tumors. In: Skin Tumors: Experimental and Clinical Aspects. Conti CJ, Slaga TJ, Klein-Szanto AJP (eds.). Raven Press, New York, pp. 19-53, 1989.
14 Scaglione-Sewell B, Abraham C, Bissonnette M, Skarosi SF, Hart J, Davidson NO, Wali RK, Davis BH, Sitrin M and Brasitus TA: Decreased PKC-α expression increases cellular proliferation, decreases differentiation, and enhances the transformed phenotype of CaCo-2 cells. Cancer Res *58*: 1074-1081, 1998.
15 Kim J, Thorne S H, Sun L, Huang B and Mochly-Rosen D: Sustained inhibition of PKCα reduces intravasation and lung seeding during mammary tumor metastasis in an *in vivo* mouse model. Oncogene *30*: 323-333, 2011.
16 Gao J, Lauer FT, Dunaway S and Burchiel SW: Cytochrome P450 1B1 is required for 7,12-dimethylbenz(a)-anthracene (DMBA) induced spleen cell immunotoxicity. Toxicol Sci *86*: 68-74, 2005.
17 DiGiovanni J: Multistage carcinogenesis in mouse skin. Pharmacol Ther *54*: 63-128, 1992.
18 Paz-Ares L, Douillard JY, Koralewski P, Manegold C, Smit EF, Reyes JM, Chang GC, John WJ, Peterson PM, Obasaju CK, Lahn M and Gandara DR: Phase III study of gemcitabine and cisplatin with or without aprinocarsen, a protein kinase C-alpha antisense oligonucleotide, in patients with advanced-stage non-small-cell lung cancer. J Clin Oncol *24*: 1428-1434, 2006.
19 Marshall JL, Eisenberg SG, Johnson MD, Hanfelt J, Dorr FA, El-Ashry D, Oberst M, Fuxman Y, Holmlund J and Malik S: A phase II trial of ISIS 3521 in patients with metastatic colorectal cancer. Clin Colorectal Cancer *4*: 268-274, 2004.

Received March 16, 2012
Revised April 24, 2012
Accepted April 26, 2012