0022-5347/03/1703-0773/0
THE JOURNAL OF UROLOGY®

Vol. 170, 773–776, September 2003
Printed in U.S.A.
DOI: 10.1097/01.ju.0000081278.64511.96

INHIBITION OF BLADDER TUMOR GROWTH BY THE GREEN TEA DERIVATIVE EPIGALLOCATECHIN-3-GALLATE

J. KARL KEMBERLING,* JAMES A. HAMPTON, RICK W. KECK, MICHAEL A. GOMEZ AND STEVEN H. SELMAN†

From the Departments of Urology (JKK, RWK, MAG, SHS) and Pathology (JAH), Medical College of Ohio, Toledo, Ohio

ABSTRACT

Purpose: We evaluated the green tea derivative epigallocatechin-3-gallate (EGCG) as an intravesical agent for the prevention of transitional cell tumor implantation.

Materials and Methods: In vitro studies were performed in the AY-27 rat transitional cell cancer and the L1210 mouse leukemia cell lines. Cells were exposed to increasing concentrations of EGCG for 30 minutes to 48 hours. Surviving cell colonies were then determined. A DNA ladder assay was performed in the 2 cell lines. Fisher 344 rats were used for in vivo studies with an intravesical tumor implantation model. Group 1 (12 rats) served as a control (tumor implantation and medium wash only). In group 2 (28 rats) 200 μM EGCG were instilled intravesically 30 minutes after tumor implantation. Rats were sacrificed 3 weeks following treatment. Gross and histological analyses were then performed on the bladders.

Results: At 6.0×10^4 cells per 100 mm dish a time dose dependent response was observed. After 2 hours of treatment with EGCG 100% cell lethality of the AY-27 cell line occurred at concentrations greater than 100 μM. Strong banding on the DNA ladder assay was seen with the L1210 mouse leukemia cell line. Only weak banding patterns were found in the AY-27 cell line treated with EGCG (100 and 200 μM) for 24 hours. All 12 controls were successfully implanted with tumors. In group 2 (EGCG instillation) 18 of the 28 animals (64%) were free of tumor (Fisher's exact test $p = 0.001$).

Conclusions: The clonal assays showed a time dose related response to EGCG. Intravesical instillation of EGCG inhibits the growth of AY-27 rat transitional cells implanted in this model.

KEY WORDS: bladder; carcinoma, transitional cell; epicatechin; rats, inbred F344; tea

Bladder cancer is a common malignancy. The majority of bladder cancers are superficial, although there is a high rate of recurrence and 13.3% of cases progress to muscular invasion.[1] The risk of recurrence after transurethral resection is more than 60% in 7 years.[2] Tumor implantation at tumor resection may account in part for the high rate of tumor recurrence.[3] A number of intravesical agents have been administered in an attempt to decrease the recurrence rate.[4] Green tea extracts have been studied extensively in several tumor models but not for their potential as intravesical chemotherapeutic agents.[5]

Green tea is one of the most popular beverages in the world. It has recently attracted attention as a natural health promoting agent. The emperor of China, Shen Nung, is credited with first describing the therapeutic effects of tea in 2737 BC.[6] Epidemiological data have been conflicting concerning green tea consumption and the cancer incidence.[7] For example, a study by Lu et al implies that tea consumption may be associated with an increased risk of bladder cancer,[8] while a study by Bianchi et al suggests that individuals consuming greater than 5 cups of tea daily may have a decreased incidence of bladder cancer.[9] While most Western populations do not consume large amounts of tea, this quantity is not uncommon in the Asian subcontinent.

Green tea is made from the leaves of Camellia sinensis and it contains higher concentrations of polyphenolic catechins than black or oolong tea. Epigallocatechin-3-gallate (EGCG) is the most abundant catechin of green tea and it has been shown to have significant antioxidant, anticarcinogenic and antimicrobial properties.[10] EGCG is water soluble with a molecular weight of 458.4 (fig. 1). Due to its structure and stereochemistry polyphenolic rings carry numerous hydroxyl groups, making it an excellent free radical scavenger.

MATERIALS AND METHODS

Cell culture. The AY-27 rat transitional cell line was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The L1210 mouse leukemia cell line was grown in Fisher's medium supplemented with 10% equine serum and used for DNA ladder assay studies. AY-27 cells (6.0×10^4) were plated onto 100 mm culture dishes and incubated for 24 hours. This cell concentration was representative of our in vivo cell tumor instillation burden. Increasing concentrations of EGCG (25 to 300 μM) in RPMI complete medium were added to cell plates for 30-minute, and 1, 2, 4, 6 and 24-hour periods, after which culture medium was removed and replaced with RPMI without EGCG. Surviving cells were incubated for 3 days to allow for colony formation and then rinsed with 10% saline, fixed with 100% methanol and stained with Giemsa stain. Colony counts were performed under 10× magnification. Experiments were repeated in quadruplicate to determine effective treatment concentrations.

DNA ladder assay. AY-27 cells were grown to approximately 70% confluency in 75 cm flasks containing RPMI complete medium before treatment with EGCG (100 or 200 μM) for 24 to 48 hours. The L1210 mouse leukemia cell line was also grown in Fisher's medium complete suspension and treated with EGCG for 24 hours. After EGCG treatment cell

Accepted for publication April 25, 2003.
Supported by the Frank D. Stranahan Endowed Chair for Oncologic Research and Geiger Cancer Research Foundation.
* Requests for reprints: Department of Urology, Medical College of Ohio, 3065 Arlington Ave., Dowling Hall 2170, Toledo, Ohio 43614-5807.
† Financial interest and/or other relationship with QLT, Inc.

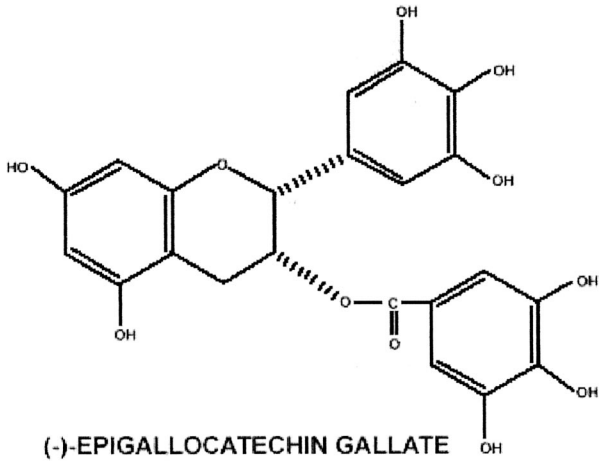

FIG. 1. Chemical structure of EGCG

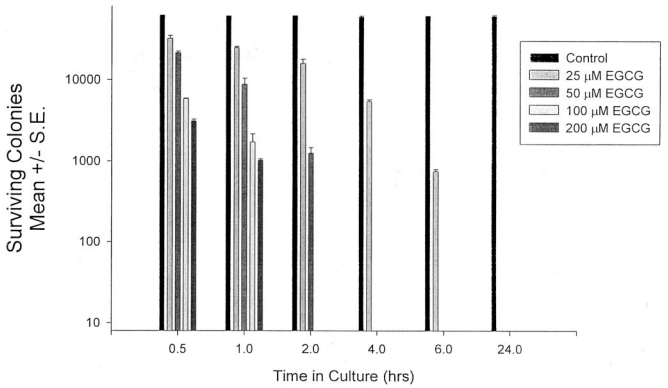

FIG. 2. In vitro AY-27 cell line showed time-dose related response to treatment at various EGCG concentrations.

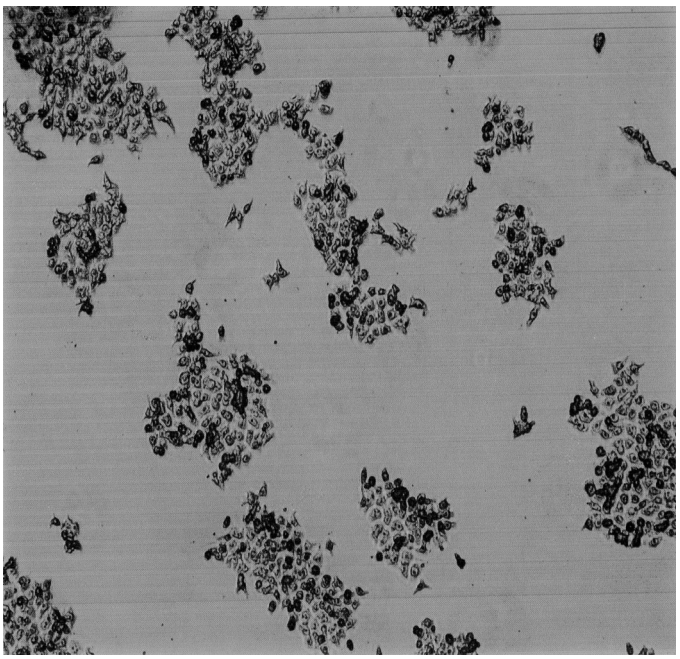

FIG. 3. Low power photomicrograph of AY-27 cells before EGCG treatment. Reduced from ×10.

lines were evaluated with a DNA ladder assay.[10] A 2% agarose gel was used with ethidium bromide stain and run for 30 minutes using a 1 kbp reference ladder.

In vivo study. Fisher 344 rats were maintained under guidelines of the animal care and use committee at our institution. A combination 80 mg/kg ketamine/12 mg/kg xylazine intraperitoneal injection was used to anesthetize the rats prior to surgery. The abdomen was shaved and prepared with an iodine solution under sterile conditions. Oxytetracycline (20 mg) was injected subcutaneously for antibacterial prophylaxis. Under 4× microscopic magnification a 1.5 cm midline incision was made to expose the bladder. A sterile 22 gauge Angiocath (Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah) cannula was then advanced transurethrally. A blunt tipped 24 gauge copper wire was then advanced through the catheter and 6 mV current was used to cauterize the bladder wall, creating a small white puckered area. The skin and muscular layers of the abdominal wall were closed with a running 4-zero silk suture.

AY-27 (10^5) cells suspended in 0.25 cc serum-free RPMI-1640 medium were instilled transurethrally into 40 rats. The catheters were then capped and tumor cells were allowed to dwell inside the bladder for 30 minutes. After tumor cell instillation 12 rats (group 1) received 2 separate hourly instillations of culture medium. The 28 other rats (group 2) were treated with a freshly prepared solution of 0.25 cc 200 μM EGCG solution in RPMI-1640 medium for 2 separate dwell times of 1 hour.

At the end of 2 hours the catheters were drained. All rats survived and were transferred to the animal care facility for recovery. The rats were sacrificed 3 weeks later. The bladders were removed, placed in 10% phosphate buffered formalin solution and allowed to fix for several hours. They were hemisected with a razor, examined under 4× magnification and sent for histopathological sectioning. Statistical analysis was performed using software (SPSS, Chicago, Illinois).

RESULTS

On clonal growth assays following exposure to EGCG the survival of AY-27 cells revealed a time dose dependent response (fig. 2). At 2 hours of exposure at EGCG concentrations of 100 μM or greater 100% cell lethality was observed (figs. 3 and 4). On light microscopy the AY-27 transitional cancer and L1210 cell lines treated for 2 hours showed morphological changes, including cellular shrinkage, pyknosis and surface blebbing (fig. 5). On DNA ladder assays the L1210 mouse leukemia cell line showed a distinct, vibrant banding pattern suggestive of apoptosis. However, AY-27 cells treated for 24 hours with EGCG (100 and 200 μM) showed a weak banding pattern (fig. 6). In the in vivo study the 12 group 1 controls demonstrated a 100% tumor growth rate (see table). The 28 group 2 EGCG treated rats had a tumor growth rate of 36% (10 of 28) (fig. 7). Statistical significance was achieved (Fisher's exact test $p = 0.001$).

DISCUSSION

Individuals in Asian cultures have believed for centuries that green tea has properties beneficial to human health. EGCG, the principal polyphenolic catechin in green tea, is inexpensive and water soluble, making it an attractive potential chemotherapeutic agent. It is readily available and easily processed from green tea leaves. In vitro studies of green tea polyphenols in various cancer cell cultures have used treatment concentrations of EGCG that are much higher than what is bioavailable with green tea consumption.[11] With direct instillation of EGCG to the tumor site we are able to bypass the effects of metabolism, digestion and excretion. To our knowledge EGCG has never been studied as an intravesical chemotherapeutic agent.

A number of antineoplastic properties of EGCG have been described, including inhibition of tumor initiation and promotion, induction of apoptosis and cell cycle arrest.[12] Morre et al observed preferential inhibition of cancer cells treated

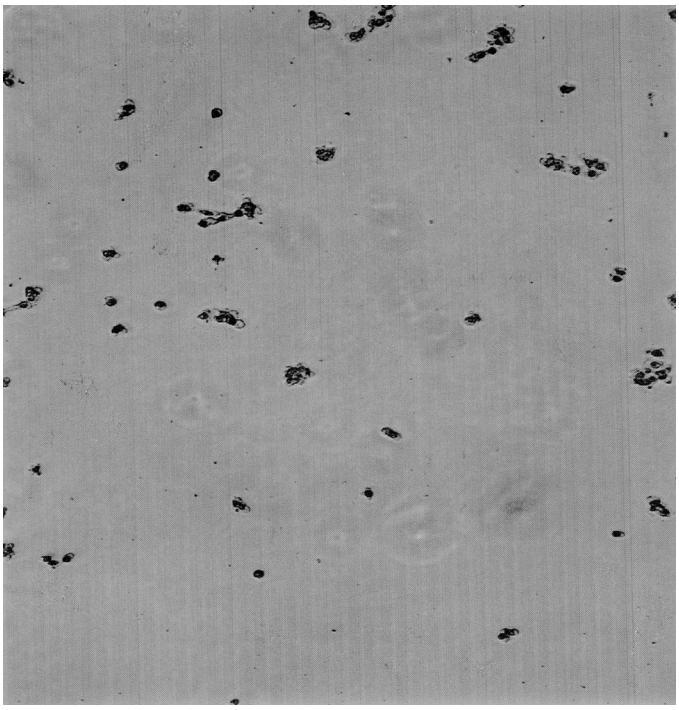

FIG. 4. Low power photomicrograph of AY-27 cells 1 hour after treatment with 200 μM EGCG. Reduced from ×10.

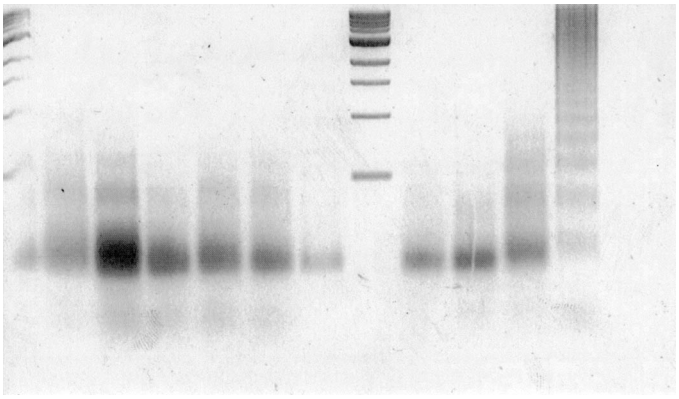

FIG. 6. DNA ladder assay of 12 wells shows 1 kbp reference ladder (lane 1), nontreated AY-27 cells (lanes 2 and 3), AY-27 cells after 24-hour 200 μM EGCG treatment (lanes 4 and 5), AY-27 cells after 24-hour 100 μM EGCG treatment (lanes 6 and 7), 1 kbp reference ladder (lane 8), nontreated L1210 cells (lanes 9 and 10), L1210 cells starved for 24 hours (lane 11) and L1210 cells after 24-hour 200 μM EGCG treatment with distinct banding pattern (lane 12).

In vivo study using intravesical instillation of 200 μM EGCG solution for 2 hours in Fisher 344 rat model

	Controls	Treated
No. rats	12	28
No. tumor (%)	12 (100)	10 (34)
% Tumor-free	0	64

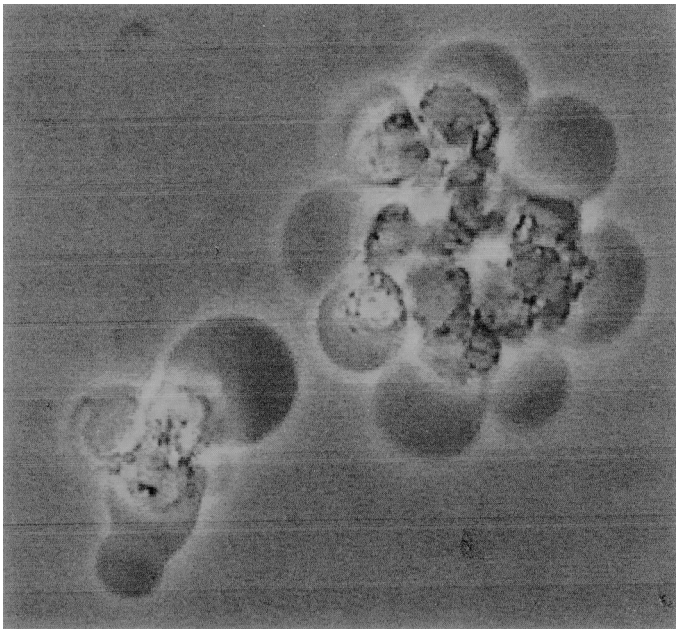

FIG. 5. Within 1 hour of adding 200 μM EGCG morphological changes occurred, including cellular shrinkage, pyknosis and surface blebbing. Reduced from ×40.

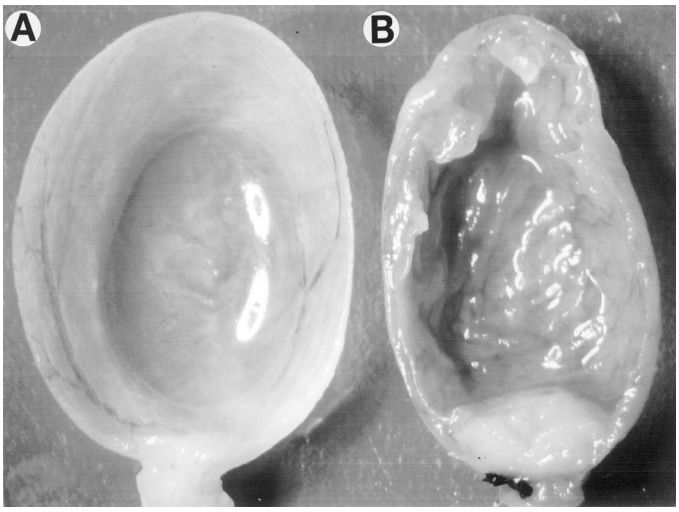

FIG. 7. *A*, rat bladder 3 weeks after intravesical EGCG treatment. *B*, bladder not treated with EGCG had visible tumor.

with EGCG that was related to suppression of nicotinamide adenine dinucleotide oxidase activity.[13] EGCG blocks the activation of transcription factor nuclear factor-κB, which induces nitric oxide synthase. Nitric oxide is known to have a role in carcinogenesis and inflammation.[14] EGCG inhibits activator protein-1 transcriptional activity and DNA binding activity.[15] AP-1 is believed to be a tumor promoter. Angiogenesis inhibition in several tumor models has also been reported.[16]

In our study no cellular survival was seen after 2 hours of treatment with all tested concentrations of EGCG. The L1210 leukemia cell line is well known for its ability to undergo apoptosis when treated with various chemical solvents. In our study it showed prominent blebbing, cellular shrinkage and nuclear condensation, suggestive of apoptosis. AY-27 cells only had a weak banding pattern on DNA ladder assay, suggesting that other mechanisms may be involved in their death. Numerous theories have been developed in an attempt to explain the action of EGCG but our results in the AY-27 cell line do not indicate that apoptosis is the primary mechanism of action.

There is no universal acceptance for the theory of tumor implantation after transurethral bladder tumor resection but several studies are in support of the theory. Weldon and Soloway noted tumor cell implantation with cauterization of the urothelium.[3] Tumors located in the bladder dome, which is an infrequent initial presentation, are thought to result from cystoscopic trauma and tumor cell implantation at the injury site.

Agents as diverse as water, thiotepa, mitomycin C, ethoglucid, doxorubicin, cisplatin and epirubicin have been studied and to various degrees each causes some decrease in the implantation rate.[4] Several studies have shown the effectiveness of immediate post-resection chemotherapy with agents such as doxorubicin and mitomycin C.[17,18] The introduction of EGCG 30 minutes after tumor introduction represents the approximate time that chemotherapy would be initiated after tumor resection. Although bacillus Calmette-Guerin is one of the most effective agents for preventing recurrence, it cannot be used immediately after resection secondary to systemic absorption. Green tea extracts, particularly EGCG, are not harmful if absorbed and they reportedly do not effect normal tissue, making it an ideal potential agent. Our in vivo data suggest that EGCG instillation decreases tumor implantation.

CONCLUSIONS

The green tea extract EGCG inhibits cellular growth of the AY-27 rat transitional cancer cell line. Instillation of 200 μM intravesical EGCG for 2 hours appears to decrease significantly the incidence of bladder tumor implantation in the Fisher 344 rat model. To our knowledge this is the first study evaluating the potential of EGCG as an intravesical chemotherapeutic agent.

The AY-27 rat transitional cell line was provided by Dr. Ronald Moore, Cross Cancer Institute, University of Alberta.

REFERENCES

1. Kiemeney, L. A., Witjes, J. A., Verbeek, A. L., Heijbroek, R. P. and Debruyne, F. M.: The clinical epidemiology of superficial bladder cancer. Dutch South-East Cooperative Urological Group. Br J Cancer, **67:** 806, 1993
2. Lamm, D. L. and Griffith, J. G.: The place of intravesical chemotherapy as defined by results of prospective randomized studies (substances and treatment schemes). Prog Clin Biol Res, **378:** 43, 1992
3. Weldon, T. E. and Soloway, M. S.: Susceptibility of urothelium to neoplastic cellular implantation. Urology, **5:** 824, 1975
4. Pode, D., Horowitz, A. T., Vlodavsky, I., Shapiro, A. and Biran, S.: Prevention of human bladder tumor cell implantation in an in vitro assay. J Urol, **137:** 777, 1987
5. Alschuler, L.: Green tea: healing tonic. Am J Natur Med, **5:** 28, 1998
6. Harbowy, M. E. and Balentine, D. A.: Tea chemistry. Crit Rev Plant Sci, **16:** 415, 1997
7. Bushman, J. L.: Green tea and cancer in humans: a review of the literature. Nutr Cancer, **31:** 151, 1998
8. Lu, C. M., Lan, S. J., Lee, Y. H., Huang, J. K., Huang, C. H. and Hsieh, C. C.: Tea consumption: fluid intake and bladder cancer risk in Southern Taiwan. Urology, **54:** 823, 1999
9. Bianchi, G. D., Cerhan, J. R., Parker, A. S., Putnam, S. D., See, W. A., Lynch, C. F. et al: Tea consumption and risk of bladder and kidney cancers in a population-based case-control study. Am J Epidemiology, **151:** 377, 2000
10. Fujiki, H., Yoshizawa, S., Horiuchi, T., Suganuma, M., Yatsunami, J., Nishiwaki, S. et al: Anticarcinogenic effects of (−)-epigallocatechin gallate. Prev Med, **21:** 503, 1992
11. Lambert, J. D. and Yang, C. S.: Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutat Res, **523-524:** 201, 2003
12. Yang, C. S., Chung, J. Y., Yang, G. Y., Li, C., Meng, X. and Lee, M. J.: Mechanisms of inhibition of carcinogenesis by tea. Biofactors, **13:** 73, 2000
13. Morre, J. D., Bridge, A., Wu, L. Y. and Morre, D. M.: Preferential inhibition by (−)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. Biochem Pharmacol, **60:** 937, 2000
14. Lin, Y. L. and Lin, J. K.: (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. Mol Pharmacol, **52:** 465, 1997
15. Dong, Z., Ma, W., Huang, C. and Yang, C. S.: Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (−)-epigallocatechin gallate, and theaflavins. Cancer Res, **57:** 4414, 1997
16. Bertolini, F., Fusetti, L., Rabascio, C., Cinieri, S., Martinelli, G. and Pruneri, G.: Inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis by green tea in animal models of human high-grade non-Hodgkin's lymphoma. Leukemia, **14:** 1477, 2000
17. Okamura, K., Ono, Y., Kinukawa, T., Matsuura, O., Yamada, S., Ando, T. et al: Randomized study of early instillation of (2″R)-4′-O-tetrahydropyranyl-doxorubicin for a single superficial bladder cancer. Cancer, **94:** 2363, 2002
18. Solsona, E., Iborra, I., Ricós, J. V., Monrós, J. L., Casanova, J. and Dumont, R.: Effectiveness of a single immediate mitomycin-C instillation in patients with low risk superficial bladder cancer: short and long-term followup. J Urol, **161:** 1120, 1999