Enhancing chemosensitivity in oral squamous cell carcinoma by lentivirus vector-mediated RNA interference targeting EGFR and MRP2

YING-JU CHEN, SHIUAN-YIN CHEN, RONALD LOVE, YI-CHU KU, YI-HUI LAI, CHIAO-LING HUNG, YU-FEN LI, YIN-CHE LU and CHIEN-KUO TAI

Department of Life Science and Institutes of Molecular Biology and Biomedical Science, National Chung Cheng University, Chia-Yi 62102; Institute of Biostatistics, China Medical University, Taichung 40402; Division of Hematology-Oncology, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chia-Yi 60002; Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, R.O.C.

Received December 4, 2014; Accepted January 14, 2016

DOI: 10.3892/ol.2016.4883

Abstract. Oral cancer is the eighth most common type of cancer among men worldwide, with an age-standardized rate of 6.3 per 100,000, and is the fourth leading cause of cancer-associated mortality among men in Taiwan. Cisplatin and 5-fluorouracil (5-FU) are two of the most frequently utilized chemotherapy drugs for the treatment of oral cancer. Although oral cancer patients initially benefit from chemotherapy with these drugs, they may develop resistance to them, which worsens their prognosis and reduces survival rates. It has been reported that increased levels of epidermal growth factor receptor (EGFR) and multidrug resistance-associated protein 2 (MRP2) induce drug resistance in numerous types of human cancer. Therefore, the present study employed lentivirus vector-mediated RNA interference (RNAi) in order to target the genes encoding EGFR and MRP2 in the oral squamous cell carcinoma cell line OC2. It was observed that RNAi-mediated downregulation of EGFR or MRP2 increased the sensitivity to 5-FU and cisplatin in OC2 cells. Downregulation of EGFR resulted in significant suppression of OC2 tumor growth following 5-FU administration. However, simultaneous downregulation of the two genes did not further suppress the tumor growth, indicating that MRP2 does not have a significant role in the chemosensitivity of EGFR-downregulated cells to 5-FU. In contrast, downregulation of MRP2 was demonstrated to significantly enhance the therapeutic effects of cisplatin in EGFR-downregulated OC2 tumors. The observation that the expression of MRP2 was positively correlated with the level of cisplatin resistance in cells suggests that RNAi-mediated downregulation of MRP2 may be applicable as a therapeutic approach toward reversing MRP2-dependent cisplatin resistance in oral cancer.

Introduction

Oral cancer is the eighth most common type of cancer among men worldwide, with an age-standardized rate of 6.3 per 100,000 (1,2), and is the fourth leading cause of cancer-associated mortality among men in Taiwan (3). In addition, oral cancer has been ranked highest with respect to the rates of incidence and mortality among men aged 25-44 years in Taiwan since 2005 (3). Chemotherapy has been used as the primary treatment for patients exhibiting recurrent or metastatic cancers, including oral cancer (4). Of the various chemotherapy drugs available, cisplatin and 5-fluorouracil (5-FU) are the two most frequently utilized for the treatment of oral cancer (5). However, the efficacy of cancer chemotherapy is often limited by the development of drug resistance by the cancer cells in clinical practice (6).

It has been demonstrated that multidrug resistance results largely from the expression of adenosine triphosphate-binding cassette (ABC) transporters with broad drug specificity (7-9). Multidrug resistance-associated protein 2 (MRP2), a member of the ABC transporter superfamily, has been observed in various tumors to confer resistance to a number of therapeutic drugs, including cisplatin (10-12). The levels of MRP2 messenger (m)RNA in certain human cancer cells are inversely correlated with their sensitivity to cisplatin (13,14). Thus, increased expression of the gene encoding MRP2 in cancer cells may reduce the intracellular accumulation of cisplatin (15). By contrast, a reduction in MRP2 expression...
using the RNA interference (RNAi) approach was demonstrated to reverse MRP2-dependent cisplatin resistance in human ovarian carcinoma (16) and nasopharyngeal carcinoma cells (17). In addition, it has been confirmed that the mRNA levels of MRP2 are markedly correlated with the half-maximal inhibitory concentration (IC50) value of 5-FU in esophageal carcinoma cells (18). Therefore, knockdown of MRP2 may influence the sensitivity of cancer cells to 5-FU.

Epidermal growth factor receptor (EGFR), which is one of most well-studied receptor tyrosine kinases, promotes cancer cell growth and is associated with drug resistance (19). Thus, it has emerged as a significant target for the development of anticancer therapy (20). Although cancer patients may initially benefit from EGFR-targeted therapies, drug resistance frequently develops due to crosstalk or inappropriate activation of downstream signaling pathways of EGFR (21). Instead of using EGFR-targeted monoclonal antibodies or small-molecule inhibitors to inhibit the activity of EGFR, the expression of its gene, EGFR, in cancer cells may be silenced using RNAi techniques (22). Therefore, lentivirus vector-mediated RNAi was used in the present study to silence the expression of EGFR and MRP2 in oral cancer. The aim of the present study was to enhance the chemosensitivity of oral cancer cells to the chemotherapeutic drugs cisplatin and 5-FU, and to reverse the drug resistance exhibited by these cells.

Materials and methods

Cell cultures. The oral squamous cell carcinoma (OSCC) cell lines OC2 and OC3L (23,24), derived from two Taiwanese male patients who demonstrated habits of alcohol drinking, betel quid chewing and cigarette smoking, were maintained in RPMI 1640 medium (catalog no., 11875-085; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; catalog no., 10437-028; Invitrogen; Thermo Fisher Scientific, Inc.). The OSCC cell lines SCC25 [obtained from American Type Culture Collection (ATCC), Manassas, VA, USA] and HSC3 (obtained from Health Science Research Resources Bank, Osaka, Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s Nutrient Mixture F-12 (1:1 mixture; catalog no., 11330-032, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS. SCC25 cells were additionally supplied with 400 ng/ml hydrocortisone (Sigma-Aldrich) for 2 days. Cells infected with shMRP2 were set to perform the following steps: For EGFR, 95°C for 5 min, followed by 30 cycles of successive incubation at 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec; for MRP2, 95°C for 5 min, followed by 38 cycles of successive incubation at 95°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec; for GAPDH, 95°C for 5 min, followed by 26 cycles of successive incubation at 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec. The PCR products and DNA ladder (catalog no., 02001-500; OmicsBio, Taipei, Taiwan) were resolved on a 2% agarose gel (catalog no., 0710-250G; Amresco, Solon, OH, USA) and visualized by ethidium bromide (catalog no., X328; Protech Technology Enterprise Co., Ltd.) staining. Expression of EGFR and MRP2 was quantified using ImageJ software v1.49 (http://imagej.nih.gov/ij/download.html; accessed October 21, 2014) and normalized against that of GAPDH.

Lentivirus vector production and transfection. Lentivirus vectors were produced by transient transfection of pCMVdeltaR8.91, pMD.G and pLKO.1-puro or pLKO-TRC008 vectors, carrying a short-hairpin RNA (shRNA), into 293T cells using calcium phosphate precipitation (2.5 M CaCl2, catalog no., 1332-01; J.T. Baker, Phillipsburg, NJ, USA); 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), catalog no., 15630-080 (Gibco; Thermo Fisher Scientific, Inc.); 2X HEPES-buffed saline, catalog no., 7021 (Sigma-Aldrich); 10 mM KCl, catalog no., 3040-01 (J.T. Baker); 1.4 mM Na2HPO4, catalog no., 0404-500G (Amresco); 240 mM NaCl, catalog no., 0241-2.5K (Amresco); 10 mM chloroquine, catalog no., c6628 (Sigma-Aldrich), as described previously (25). pLKO plasmids were obtained from the National RNAi Core Facility at the Academia Sinica in Taipei, Taiwan. The shRNA sequences specific for EGFR and MRP2 were 5’-GCTGGATGATAGCGCGATA-3’ and 5’-GCCGGTGTCAGATTATCATCT-3’, respectively. OSCC cells infected with shRNA-expressing lentivirus vectors (shEGFR or shMRP2) were selected by incubation with 2 μg/ml puromycin (Sigma-Aldrich) or 20 μg/ml DEAB (Sigma-Aldrich) for 2 days. Cells infected with sh-red fluorescent protein (RFP) were used as a control.

Western blot analysis. Cells were lysed by the treatment of RIPA Lysis and Extraction Buffer (catalog no., 89900; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The cell lysates were suspended in sodium dodecyl sulfate (SDS) gel-loading buffer (62.5 mM Tris-HCl (pH 6.8); 2% SDS; 10% glycerol; 100 mM dithiothreitol; 0.01% bromophenol blue) and boiled for 5 min. Protein samples were run on SDS-10% polyacrylamide gels with protein marker
Cell proliferation analysis by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. To determine the proliferation rate of cells transfected with shEGFR, the cells were seeded onto replicate 96-well plates (catalog no., 300096; SPL Life Sciences, Co., Ltd., Gyeonggi, Korea) at a density of 400 cells/well. Following overnight culture, cell proliferation was determined daily by MTS assay, using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA).

Colony formation assay. A total of 400 cells were seeded onto 6-well plates (catalog no., 30006; SPL Life Sciences, Co., Ltd.) and cultured for 1-2 weeks or until colonies of cells were visible with the naked eye. Cell colonies were stained with 0.4% crystal violet (Sigma-Aldrich) in 50% methanol (catalog no., 322415; Sigma-Aldrich) for 10 min, followed by washing with phosphate-buffered saline (PBS), air-drying at room temperature for 2 days and counting with the naked eye. Experiments were performed in triplicate.

Determination of the IC_{50} values of drugs. To determine the IC_{50} values of 5-FU (catalog no., F6627; Sigma-Aldrich) and cisplatin (catalog no., P4394; Sigma-Aldrich), cells were seeded onto replicate 96-well plates at a density of 2,000 cells/well. Following overnight culture, the cells were incubated with 5-FU or cisplatin at various concentrations (0.00, 0.25, 0.50, 1, 2, 4, 6, 8, 10, 12, 14 and 16 µg/ml) for 3 days, and the remaining viable cells were determined by MTS assay.

Apoptosis analysis. OC2 and OC2 cisplatin-resistant (CisR) cells were exposed to increasing concentrations of cisplatin, ranging from 1-16 µg/ml for 24 h. The cells were subsequently harvested with trypsin (catalog no., 25200-072; Gibco; Thermo Fisher Scientific, Inc.), washed with PBS and resuspended in binding buffer (catalog no., AVF250, Strong Biotech Corp., Taipei, Taiwan) containing the optimal concentration of calcium required for Annexin V to bind to phosphatidylinerine on the cell surface. Annexin V-fluorescein isothiocyanate (Strong Biotech Corp.) and propidium iodide (PI; Sigma-Aldrich) were added to the cell suspension and allowed to incubate in the dark for 15 min at room temperature. The cells were subsequently analyzed by flow cytometry using a FACSCalibur™ with BD CellQuest™ Pro Software version 6.0 (BD Biosciences, San Jose, CA, USA), and the percentages of early (Annexin V+, PI−) and late apoptotic cells (Annexin V+, PI+) were calculated according to the manufacturer's instructions.

Development of the OC2CisR cell line. OC2 cells were seeded onto 6-cm culture plates (catalog no., 20101, SPL Life Sciences, Co., Ltd.) at 50% confluency. Following overnight culture, the cells were incubated with cisplatin at a concentration of 0.10 µg/ml until the cultures approached confluency. The cells were subcultured onto another 6-cm plate at 50% confluency and incubated with cisplatin at a concentration of 0.25 µg/ml. Following the repetition of the procedure with the increased concentrations of 0.50 and 0.75 µg/ml, the cells were able to survive in a medium containing 0.75 µg/ml cisplatin and the cells were designated as the OC2CisR cell line.

In vivo experiments. OSCC cells were implanted into the right dorsal flank of athymic female BALB/c nude mice (randomly divided into groups, n=6/group; National Laboratory Animal Center, Taipei, Taiwan). When the tumor volumes reached ~100 mm³, the mice were administered intraperitoneal injections of 5-FU (15 mg/kg/day) for a total of 6 treatments, or cisplatin at a weekly dose of 3 mg/kg for a total of 3 treatments. The tumor volumes were measured daily, and the maximum allowable size of the tumors was 15 mm in diameter. The housing conditions of the mice were as follows: 12-h light/12-h dark cycle; temperatures of 18-23°C with 40-60% humidity; and water and food accessible at all times. All experiments were performed under license from the Institutional Animal Care and Use Committee of the National Chung Cheng University.

Statistical analysis. Student's t-tests were performed for statistical analysis of relative gene expression, cell proliferation, cell apoptosis, colony-formation, IC_{50} value and relative tumor volume. All data are presented as the mean ± standard error (SE). For the comparison of tumor growth rates, generalized linear models with generalized estimated equations were used to account for the correlation between the repeated measurements. A significant interaction between time and group in the generalized linear models meant that the growth rates of the groups were changing over time in various ways. A P-value of <0.05 was considered to indicate a statistically significant difference. Analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

Results
Expression levels of EGFR and MRP2 in OSCC cell lines. RT-PCR was employed to examine the expression of EGFR and MRP2 in the OSCC cell lines OC2, OCSL, SCC25 and HSC3. Fig. 1 shows the differential expression of these two genes among the four cell types. With respect to the two OSCC cell types derived from Taiwanese male patients who habitually drank alcohol, chewed betel-quin and smoked cigarettes, namely, OC2 and OCSL, the former exhibited increased expression of EGFR and MRP2 compared with the latter (P<0.05). Thus, OC2 was selected as the target cell line for evaluation of RNAi-mediated downregulation of EGFR and MRP2.
Downregulation of EGFR expression inhibits the growth of OC2 cells. Western blot analysis was utilized to determine the expression of EGFR protein in OC2 cells transfected with shEGFR, thus confirming the specificity of RNAi-mediated silencing of EGFR. It was observed that shEGFR transfection effectively reduced the expression of EGFR protein in OC2 cells (Fig. 2A). In addition, MTS and colony formation assays were employed to investigate the proliferation rate and colony-forming ability, respectively, of the transfected OC2 cells. A significant difference was observed between the growth of shEGFR- and shRFP-transfected OC2 cells (P<0.01; Fig. 2B and C), indicating that downregulation of EGFR expression inhibited the growth of the OC2 cells.

Downregulation of EGFR and MRP2 enhances the sensitivity of OC2 cells to 5-FU. OC2 cells transfected with shMRP2 alone or in combination with shEGFR were established to determine whether MRP2 downregulation enhanced the chemosensitivity of OC2 cells to 5-FU. Fig. 3A shows the reduced expression of EGFR and MRP2 in OC2/shEGFR/shMRP2 cells compared with the control group OC2/shRFP (P<0.01). Transfection of OC2 cells with shEGFR was effective at sensitizing the cells to 5-FU according to the IC_{50} value, which was determined using the MTS assay (Fig. 3B). In addition, downregulation of MRP2 increased the sensitivity of the cells to 5-FU. However, knockdown of MRP2 and EGFR did not further decrease the IC_{50} value of 5-FU, compared with the cells transfected with shEGFR alone.

Mice bearing subcutaneous OC2 tumors were treated with 5-FU to determine whether the RNAi-induced downregulation of EGFR and MRP2 expression confers a therapeutic benefit in vivo (Fig. 3C). Downregulation of EGFR resulted in a significant suppression of tumor growth following 5-FU administration, compared with the control group (OC2/shRFP; Fig. 3D). However, downregulation of MRP2 and EGFR did not further suppress tumor growth, indicating that MRP2 does not have a significant role in the chemosensitivity of EGFR-downregulated OC2 cells to 5-FU.

Development of the OC2CisR cell line. Previous studies have demonstrated that MRP2 confers resistance to cisplatin in certain types of cancer (9-11). A cisplatin-resistant OC2 cell line (designated OC2CisR) was generated for use as the target cell for subsequent experiments, in order to determine...
the role of MRP2 in OSCC. These cells were generated by treating OC2 cells with cisplatin at progressively increasing concentrations (0.10, 0.25, 0.50 and 0.75 µg/ml) until they were able to survive in a medium containing 0.75 µg/ml cisplatin. The expression of EGFR and MRP2 in OC2 and OC2CisR cells was determined using RT-PCR. MRP2 expression was significantly increased in OC2CisR cells, compared with OC2 cells (P<0.05), whereas EGFR expression did not differ significantly between the two cell lines (Fig. 4A). Cell viability analysis confirmed that OC2CisR cells exhibited an increased resistance to cisplatin, compared with OC2 cells (P<0.05; Fig. 4B). A cell apoptosis assay additionally confirmed that the OC2CisR cells were more resistant to the killing effect of cisplatin, compared with the OC2 cell line (Fig. 4C).

Furthermore, mice bearing OC2 and OC2CisR subcutaneous tumors were treated with cisplatin to evaluate whether OC2CisR cells were more resistant to cisplatin, compared with OC2 cells in vivo (Fig. 4D). It was observed that the growth rate of OC2CisR subcutaneous tumors was significantly higher than that of OC2 subcutaneous tumors in the mice following cisplatin administration (Fig. 4E). The tumor growth rate was 52.1% higher per day in the OC2CisR group, compared with the OC2 group (SE=23.0%; P=0.024). These observations confirmed that OC2CisR cells are more resistant to cisplatin, compared with OC2 cells in vitro and in vivo, suggesting that elevated expression of MRP2 in OC2CisR cells contributed to their resistance to cisplatin.

Downregulation of EGFR and MRP2 increases the inhibitory effects of cisplatin on the growth of OC2CisR tumors. OC2CisR cells were transfected with shMRP2 and shEGFR to establish whether MRP2 had a significant role in cisplatin resistance in these cells, and to observe whether downregulation...
Figure 4. Development of the OC2CisR cell line. (A) Expression of EGFR and MRP2 in OC2CisR cells. The messenger RNA levels of EGFR and MRP2 in OC2CisR cells were quantified using reverse transcription-polymerase chain reaction analysis. The expression levels of EGFR and MRP2 in OC2 cells were assigned a value of 100% as controls. (B) Determination of the IC50 values of cisplatin in cells. The IC50 value of cisplatin in OC2 was used as the control. (C) Cell apoptosis assay. OC2 and OC2CisR cells were exposed to increasing concentrations of cisplatin ranging from 1 to 16 µg/ml for 24 h, and stained with Annexin V-fluorescein isothiocyanate and propidium iodide, followed by flow cytometric analysis. The percentage of total apoptotic cells is shown. (D) Diagram illustrating the procedure of the in vivo experiments. A total of 8x10^6 cells were subcutaneously implanted into the right dorsal flank of nude mice (n=6/group). When the tumor volumes reached ~100 mm³ (set as day 0), the mice were administered intraperitoneal injections of cisplatin (3 mg/kg) for a total of 3 treatments. The tumor volumes were measured daily. Solid arrow indicates cisplatin treatment on days 0, 7 and 14. (E) Comparison of tumor growth rates in the mice following cisplatin administration. The tumor volumes on day 0 were assigned a value of 100%. The interaction of time and group in the generalized linear model with generalized estimated equation was 52.1% (P=0.024), which indicated that the linear growth rate was 52.1% higher per day for the OC2CisR group compared with the OC2 group. Data are presented as the mean ± standard error.

Figure 5. Downregulation of EGFR and MRP2 increases the inhibitory effects of cisplatin on the growth of OC2CisR tumors. (A) Reduced expression of EGFR and MRP2 in OC2CisR/shEGFR/shMRP2 cells. The messenger RNA levels of EGFR and MRP2 in cells were quantified using reverse transcription-polymerase chain reaction analysis. The expression levels of EGFR and MRP2 in OC2/shRFP were assigned a value of 100% as controls. (B) Therapeutic effect achieved by the downregulation of EGFR and MRP2 in OC2CisR tumors. The procedure of the in vivo experiments is illustrated in Fig. 4D (n=6/group). The tumor volumes on day 0 were assigned a value of 100%. The interaction of time and group in the generalized model with generalized estimated equation was 37.4% (P=0.035), which indicated that the linear growth rate was 37.4% higher per day for the OC2CisR/shEGFR/shMRP2 group compared with the OC2CisR/shEGFR/shMRP2 group. Data are presented as the mean ± standard error. *P<0.05. EGFR, epidermal growth factor receptor; MRP2, multidrug resistance-associated protein 2; IC50, half-maximal inhibitory concentration; s.c., subcutaneously; i.p., intraperitoneal.
of MRP2 was able to enhance the therapeutic effects of cisplatin in EGFR-downregulated cells. Fig. 5A demonstrates the RNAi-mediated downregulation of EGFR and MRP2 expression in OC2CisR/shEGFR/shMRP2 cells in vitro, compared with the control group OC2CisR/shRFP (P<0.05). In vivo, the growth rate of OC2CisR/shEGFR/shMRP2 subcutaneous tumors was significantly lower, compared with that of OC2CisR/shEGFR subcutaneous tumors in the mice following cisplatin administration (Fig. 5B). The tumor growth rate was 37.4% lower per day in the OC2CisR/shEGFR/shMRP2 group, compared with the OC2CisR/shEGFR group (SE=17.8%; P=0.035). These results confirmed that MRP2 overexpression confers resistance to cisplatin in OC2CisR cells, and that downregulation of MRP2 is able to significantly enhance the therapeutic effects of cisplatin in EGFR-downregulated cells.

Discussion
Overexpression of EGFR is frequently observed in numerous types of cancer, including OSCC (26,27). EGFR-targeted therapies, including gefitinib (Iressa®; AstraZeneca, London, UK) (26,28-30) and cetuximab (Erbitux®; Eli Lilly and Company, Indianapolis, IN, USA) (31,32), have been studied extensively as potential methods of blocking the cancer-promoting functions of EGFR in OSCC. However, although it has been reported that EGFR inhibitors are initially beneficial for the treatment of cancer, resistance to the inhibitors eventually develops (21,33). Therefore, an alternative strategy was investigated in the present study, in which rather than targeting the EGFR protein, the expression of its gene, EGFR, in OSCC was downregulated using RNAi techniques. The results of the present study demonstrate that reduction of EGFR expression inhibits the growth and enhances the cytotoxicity of 5-FU in OSCC cells.

Although MRP2 downregulation also increased the sensitivity of OC2 cells to 5-FU, it did not contribute to the suppression of cisplatin-resistant OC2 tumors following 5-FU administration. Therefore, the ability of MRP2 downregulation to achieve further suppression of EGFR-downregulated OC2 tumors in vivo during cisplatin administration was investigated. Following establishment of the cisplatin-resistant cell line OC2CisR, it was observed that the expression of MRP2 was positively correlated with the level of cisplatin resistance in the cells, whereby the OC2CisR cells became highly resistant to the killing effect of cisplatin, compared with the parental cell line OC2. These results imply that MRP2 may have a significant role in modulating the response of OC2CisR cells to cisplatin. Furthermore, downregulation of MRP2 significantly enhanced the therapeutic effect of cisplatin in EGFR-downregulated cells.

In conclusion, MRP2-targeted therapy may be a promising strategy for overcoming the resistance to cisplatin in OSCC. Inhibitors of ABC transporters, including MRP2, have been extensively investigated in previous studies (34). However, there has been little impact of these studies on clinical outcomes (35,36). Therefore, an RNAi-mediated targeting strategy was applied in the present study, with a view to reversing multidrug resistance in tumors. Due to its specificity and effectiveness, RNAi-mediated downregulation of MRP2 may be applicable as a therapeutic approach toward reversing MRP2-dependent cisplatin resistance in OSCC.

Acknowledgements
The present study was supported by the Ditmanson Medical Foundation Chia-Yi Christian Hospital (Chia-Yi, Taiwan; grant no. R100-3) and the National Science Council of Taiwan (Taipei, Taiwan; grant no. NSC99-2320-B-194-004-MY3).

References


