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Expression of Drug Resistance-related Genes in Head and Neck Squamous Cell Carcinoma and Normal Mucosa

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We examined the expression levels of mRNA for multidrug resistance 1 (*MDR1*), multidrug resistance-associated protein (*MRP*), human canalicular multispecific organic anion transporter (cMOAT), lung resistance-related protein (*LRP*), topoisomerase IIα, β (*Topo IIα, β*) and topoisomerase I (*Topo I*) genes in human head and neck squamous cell carcinoma (HNSCC) specimens and mucosa (HNM) specimens, to elucidate their roles in relation to the biological characteristics and drug resistance *in vivo*. Fifty-eight samples (45 head and neck carcinomas and 13 head and neck mucosa) obtained during surgical resection or biopsy from 38 patients were analyzed using the quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. *MDR1, MRP, LRP, Topo IIα, Topo IIβ,* and *Topo I* gene transcripts were detected in all the samples tested, but cMOAT mRNA was not detected in them. Comparisons of the expression levels in HNSCC with those in HNM showed that the *Topo IIα* gene expression level was higher in HNSCC than in HNM (P=0.0298). Moreover, the Topo IIα mRNA level was significantly higher in metastatic lymph node samples of HNSCC than in HNM samples (P=0.0205). There were no significant differences in the six genes' expression levels between samples exposed to platinum drugs and those not exposed to platinum drugs. These results suggest that it may be effective in anticancer therapy to use topoisomerase-targetting drugs against HNSCC, especially metastatic neck tumors, and that the expression of these genes in HNSCC is not associated with platinum drug exposure.

Key words: DNA topoisomerase II — Drug resistance-related gene — Head and neck squamous cell carcinoma

Chemotherapy has become a very important option for the treatment of head and neck squamous cell carcinomas (HNSCC) in order to preserve the patient's physical functions. However, some advanced, recurrent or metastatic HNSCCs have multi-drug resistance. Drug resistance may be caused by altered membrane permeability, changes in the host's drug metabolism and detoxifying pathways, and/or alterations in DNA replication-related enzymes.

A P-glycoprotein encoded by the multidrug resistance 1 (*MDR1*) gene and a multidrug resistance-associated protein encoded by the multidrug resistance-associated protein (*MRP*) gene function as transmembrane drug efflux pumps.[1–4] The human canalicular multispecific organic anion transporter (*cMOAT*) gene is a newly discovered member of the ABC transporter superfamily and has been suggested to participate in platinum drug transport.[5] Furthermore, the *LRP* (lung resistance-related protein) gene, encoding for the human vaults protein, may mediate nucleocytoplasmic and vesicular transport of drugs.[6] It is speculated that overexpression of these genes causes cancer cells to become resistant to anticancer drugs.[3–9]

DNA topoisomerases are nuclear enzymes that play essential roles in DNA replication, transcription, chromosome segregation, and DNA recombination.[10] All cells have two major forms of topoisomerases, i.e. type I (Topo I) and type II (Topo II), and Topo I or Topo II induce single or double strand breaks, respectively.[10–12] There are two isoforms of Topo II, a 170-kDa isoform (Topo IIα) and a 180-kDa isoform (Topo IIβ), which may have different functions due to the difference in their expression during cell-cycle progression[10, 13] and their differential distribution.[14] Topoisomerases have been shown to be targets for clinically important anticancer agents.[10, 15] It was also reported that *Topo IIα* or *Topo I* gene expression increased markedly in malignant tumors.[16–21] Furthermore, it has recently been demonstrated that MDR1 and MRP play important roles in resistance to Topo II inhibitors such as etoposide or adriamicin.[8, 22, 23]

Several studies have examined drug resistance in clinical specimens of the lung,[17–19, 24–27] kidney or bladder,[28, 29] breast[30, 31] and ovary[16, 32, 33] using immunohistochemistry or molecular biological methods. However, it remains to be elucidated whether these factors are related to the drug resistance of head and neck carcinomas. In order to clarify the roles of these drug resistance-related genes in the char-

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acteristics of tumors, we analyzed the expression levels of mRNA for MDR1, MRP, cMOAT, LRP, Topo IIα, Topo IIβ and Topo I in HNSCC specimens in comparison with those in head and neck mucosa (HNM) specimens.

MATERIALS AND METHODS

Patients and samples Fifty-eight samples (45 head and neck squamous cell carcinomas and 13 head and neck mucosa) from 38 patients admitted to Hiroshima University Hospital between April 1997 and August 1998 were studied. Fresh specimens of HNSCC and HNM were obtained during surgical resection or biopsy after informed consent had been obtained. The tissues were frozen in liquid nitrogen and stored at −80°C until analyzed.

Reverse transcription-polymerase chain reaction (RT-PCR) Total cellular RNA was extracted using the guanidinium isothiocyanate-phenol method, and cDNA was synthesized using a random hexamer (Amersham, Buckinghamshire, UK) with Superscript RNase H-reverse transcriptase (GIBCO-BRL, Bethesda, MD), as described previously.[24]

PCR amplification The reverse-transcribed cDNA from each sample was subjected to PCR amplification using primers based on the *MDR1, MRP, cMOAT, LRP, Topo IIα Topo IIβ, Topo I,* and *β-actin* (internal control) gene sequences. After pre-denaturation at 94°C for 5 min, the cDNA was added to 5 μl of PCR mixture, comprising 1 μl of 10× PCR buffer (100 m*M* Tris-HCl, pH 9.0, 500 m*M* KCl), 1 μl of 15 m*M* $MgCl_2$, 2 μl of distilled water, 0.2 μl of 20 m*M* dNTPs (Takara, Tokyo), 0.2 μl of 50 μ*M* forward primer, 0.2 μl of 50 μ*M* backward primer, and 0.4 μl (0.2 U) of *Taq* polymerase (Promega, Madison, WI). The sequences of the primers used were as follows: MDR1 forward 5′-CTAATAAGAAAAAGATCAACT-3′ and reverse 5′-GGCTAGAAACAATAGTGAAAACAA-3′[7,8]; MRP forward 5′-TGGGACTGGAATGTCACG-3′ and reverse 5′-AGGAATATGCCCCGACTTC-3′[24]; cMOAT forward 5′-CTAATCTAGCCTACTCCTGC-3′ and reverse 5′-CTGCAGCTCTCTCTTCATGTGC-3′[24]; LRP forward 5′-TTCTGGATTTGGTGGACGC-3′ and reverse 5′-ACTTCTCTCCCTTGACCAC-3′[27]; Topo IIα forward 5′-ACCATTGCAGCCTGTA-3′ and reverse 5′-GCTCTTCCCATATTATCC-3′[18]; Topo IIβ forward 5′-ACAGGTGGTCGTAATG-3′ and reverse 5′-GTTTCACTGATACACC-3′[18]; Topo I forward 5′-GGAGAGACCTGAAAAGTGCTAA-3′ and reverse 5′-TAAATCTTCTCAATTGGGAC-3′.[19] Amplification was carried out using a thermal cycler (Geneamp PCR System 2400; Perkin Elmer Applied Biosystems Division, Norwalk, CO) under the following conditions: denaturation (94°C, 30 s), annealing (55°C, 30 s for MDR1; 60°C, 30 s for MRP; 64°C, 30 s for cMOAT); 65°C, 30 s for LRP; 52°C, 30 s for Topo IIα and Topo IIβ; 59°C, 30 s for Topo I), extension (72°C, 1 min) followed by a final incubation at 72°C for 7 min. In order to determine the optimal number of amplification cycles, the accuracy of the quantitative PCR procedure was tested in a titration experiment as described previously.[24] The optimal number of cycles for MDR1, MRP, cMOAT and Topo I was 24, for LRP, 28 and for Topo IIα and Topo IIβ, 25. The PCR products were 243, 293, 275, 285, 588, 583 and 247 base pairs (bp) long, corresponding to MDR1, MRP, cMOAT, LRP, Topo IIα, Topo IIβ and Topo I cDNA respectively. We used the *β-actin* gene as an internal control. The sequences of its primers were: forward 5′-AAGAGAGGCATCCTCACCCT-3′ and reverse 5′-TACATGGCTGGGGTGTTGAA-3′. The PCR conditions were as follows: denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min) for 20 amplification cycles, followed by a final incubation at 72°C for 7 min. The PCR products were 218 bp long. We found that expression levels of *β-actin* gene from all samples were similar as evaluated by ethidium bromide staining, and therefore, we concluded that the quality of harvested RNA from our samples was acceptable for molecular analysis as described previously.[24]

Quantitative analysis of PCR products and analysis of mRNA expression The PCR products were electrophoresed with 2% (w/v) agarose gels, transferred to nylon membranes (Hybond N^+; Amersham) and subjected to hybridization analysis with ^{32}P-labeled cDNA probes using procedures described previously.[24] Each filter was washed and the radioactivity level was measured with a laser imaging analyzer (BAS-2000; Fuji Photo Film, Tokyo). The PCR products of MRP, cMOAT, Topo IIα and Topo IIβ described above were used as cDNA probes, and we designed and synthesized PCR products for the MDR1 and LRP probes with the following sequences: MDR1 forward primer 5′-AAGCTTAGTACCAAAGAGGCTATG-3′, and reverse primer as above, LRP forward primer as above, and reverse primer 5′-TCCACTTTGGCAGATGGCAC-3′. We used the PCR products for Topo I and β-actin probes with the following sequences: Topo I forward primer 5′-GAAGTCCGGCATGATAACAA-3′, and reverse primer 5′-GTTTGTTAAGACTTGCTGCC-3′, β-actin forward primer, as above, and reverse primer 5′-AATGGTGATGACCTGGCCGT-3′ as described previously.[24] The radioactivity associated with gene expression in each sample was expressed as the yield of the target gene relative to that of the *β-actin* gene.

Statistical analysis The statistical significance of differences between the expression levels of each mRNA in tissue samples was analyzed with the Mann-Whitney *U*-test. All the gene expression levels were skewed toward higher expression, and were subjected to logarithmic transformation so that they approximated more closely to a normal distribution. The statistical calculations and tests were performed using Stat View J 4.11 software (Abacus, CA) and

a Macintosh computer. All the statistical tests were two-sided, the data were expressed as medians and ranges, and differences in *P*-values of less than 0.05 were considered to be significant.

RESULTS

Patients' characteristics We analyzed 58 samples (45 HNSCC and 13 HNM) from 38 patients using the RT-PCR methods. The patients' characteristics are presented in Table I. There were 30 men and 8 women. There were 29 men and 7 women in the HNSCC group, and 9 men and 3 women in the HNM group. They ranged in age from 40 to 88 years old in the HNSCC, and from 36 to 88 years old in the HNM. Twenty-six samples had been exposed to platinum drugs, and 22 samples had been treated with radiation therapy. Thirty-seven samples were obtained from patients with a primary site in HNSCC, and 8 samples were obtained from patients with metastatic cervical lymph nodes in HNSCC. Twenty-nine samples were obtained at diagnostic biopsy, and 29 samples were obtained from surgery.

Expression levels of the seven genes *MDR1*, *MRP*, *LRP*, *Topo IIα*, *Topo IIβ*, and *Topo I* gene transcripts were detected in all the samples tested, but *cMOAT* gene transcript was not detected in any of them. Representative autoradiographs of RT-PCR for the 7 genes, and for *β-actin* gene in HNSCC and HNM samples are shown in Fig. 1. There was considerable variation among tumors and normal mucous membrane tissues in the expression levels of the six genes. The median and range expression levels of the six genes except *cMOAT* gene are summarized in Table II. The expression levels of Topo IIα mRNA in HNSCC were significantly higher than those in HNM (P=0.0298, Table II). On the other hand, the differences in the expression levels for the other five genes in HNSCC and HNM were not significant (Table II). Then, we compared the levels of expression of Topo IIα mRNA among the metastatic lymph node samples, primary carcinoma samples and normal mucosal samples. The median expression levels of Topo IIα were 0.625 (0.121–2.542) in the metastatic lymph node samples (N=8), 0.351 (0.011–2.848) in the primary carcinoma samples (N=37) and 0.136 (0.006–0.694) in the normal mucosal samples

Table I. Characteristics of Patients

All patients	38
Age (years)	36–88
Sex (male/female)	30/8
All samples	58
HNSCC[a)]	45: Age 40–88 Sex (male/female) 29/7
Site	primary tumor 37; maxillary sinus 9, epipharynx 3, nasal cavity 3, buccal mucosa 3, oropharynx 2, tongue 4, floor of mouth 1, hypopharynx 8, larynx 4
	metastatic cervical lymph nodes 8
HNM[b)]	13: Age 36–88 Sex (male/female) 9/3
Site	maxillary sinus 2, hypopharynx 4, buccal mucosa 2, larynx 1, nasal cavity 2, tongue 1, epipharynx 1
Samples exposed to platinum drugs (HNSCC/HNM)	19/7

a) HNSCC, head and neck carcinoma.
b) HNM, head and neck mucosa.

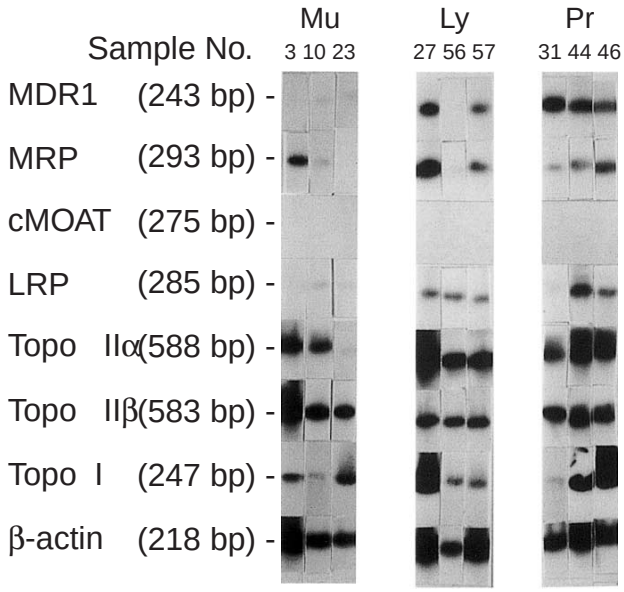

Fig. 1. Expression levels of the *MDR1*, *MRP*, *cMOAT*, *LRP*, *Topo IIα*, *Topo IIβ* and *Topo I* genes in HNSCC and HNM. Mu, head and neck mucosa samples; Ly, metastatic lymph node samples; Pr, primary carcinoma samples.

Table II. Median Values of the Six Genes' Expression in HNM and HNSCC

	HNM (N=13)	HNSCC (N=45)	P-value
MDR1	0.019 (0.02–0.639)[a)]	0.033 (0.01–0.973)	NS
MRP	0.043 (0.02–0.510)	0.105 (0.0003–1.150)	NS
LRP	0.059 (0.002–0.517)	0.090 (0.002–2.019)	NS
Topo IIα	0.136 (0.006–0.694)	0.370 (0.011–2.848)	0.0298
Topo IIβ	0.065 (0.001–0.893)	0.201 (0.0002–1.644)	NS
Topo I	0.061 (0.012–0.143)	0.071 (0.000–0.449)	NS

a) Median (range) Mann-Whitney *U*-test *P*-values <0.05 were considered to be significant.
NS, not significant.

Table III. Median Values of *Topo IIα* Gene Expression in Normal Mucosa, Metastatic Lymph Nodes and Primary Site

Mu[a] (*N*=13)	Ly[b] (*N*=8)	Pr[c] (*N*=37)
0.136[d]	0.625	0.351
(0.006–0.694)[e]	(0.121–2.542)	(0.011–2.848)

Mu vs. Ly, $P=0.025$; Ly vs. Pr, $P=0.13$ (NS); Mu vs. Pr, $P=0.0616$ (NS).
a) Mucosa samples.
b) Metastatic lymph node samples.
c) Primary carcinoma samples.
d) Medians.
e) Ranges.
P-values <0.05 were considered to be significant.
NS, not significant.

(HNM) (*N*=13) (Table III). The expression levels in the metastatic lymph nodes were significantly higher than those in the HNM ($P=0.0205$), and the expression levels in the primary carcinoma samples were relatively higher than those in the normal mucosal samples ($P=0.0616$) (Table III). In contrast, there was no difference between the expression levels in the metastatic lymph node samples and those of the primary carcinoma samples ($P=0.1300$) (Table III). The differences in the expression levels for the other five genes among the metastatic lymph node samples, primary carcinoma samples and normal mucosal samples were not significant (data not shown). We also compared the expression levels of these six genes between samples exposed to platinum drugs and those not exposed to platinum drugs. There were, however, no differences between the expression levels of these six genes in the previously treated and non-treated groups in both HNSCC and HNM (data not shown).

DISCUSSION

Topoisomerases are the targets of several anticancer drugs, but there has been no report on topoisomerases in head and neck carcinomas. This paper is the first, as far as we know, to provide detailed data about the steady-state levels of mRNA for Topo IIα, Topo IIβ and Topo I in head and neck clinical carcinoma specimens based on molecular-biological methods. Our data showed that the level of Topo IIα expression in HNSCC was significantly higher than that in HNM, and furthermore, that the level of Topo IIα expression in metastatic lymph node samples was significantly higher than that in HNM, while the expression level in the primary carcinoma samples was relatively higher than that in HNM, whereas no such difference was observed for Topo IIβ and Topo I.

A higher expression level of Topo IIα in tumors compared with normal tissues has been reported in lung cancer,[17–19] ovarian cancer[16, 33] and breast cancer.[34] High expression levels of the α isoform were seen in rapidly proliferating cells, in contrast to the β isoform, which did not vary in resting cells or through the cell cycle.[13] Moreover, Giaccone *et al*. reported that higher expression levels of Topo IIα were associated with higher expression of Ki-67, a cell proliferation marker, whereas no correlation was found between expressions of Ki-67 and Topo IIβ or Topo I in non-small cell lung cancer.[17] Furthermore, it was demonstrated that increased *Topo II* gene expression showed a strong positive correlation with cell sensitivity to Topo II inhibitors in lung cancer cell lines.[20] In fact, small cell lung cancer (SCLC) is more sensitive to Topo II inhibitors than non-small cell lung cancer (NSCLC) in clinical situations, and Syahruddin *et al*. demonstrated that the expression levels of the *Topo IIα* gene in SCLC were significantly higher than those in NSCLC.[18] Thus, it appears that Topo II inhibitors can be selective for tumors with higher Topo IIα expression, assuming that Topo IIα is the principal target enzyme. On the other hand, Yamazaki *et al*. reported that the sensitivity of lung cancer cell lines to Topo II inhibitors can not be explained by the Topo IIα content levels or Topo II catalytic activity.[35] Sandri *et al*. indicated that Topo IIβ may play a significant role as a target for anti-tumor therapy, because the Topo IIβ protein is more widely expressed than the Topo IIα protein in breast cancer cells.[30] Further studies are required to determine whether the level of Topo IIα and/or Topo IIβ expression can be good markers of sensitivity to Topo II inhibitors. Furthermore, our findings suggest that although Topo II inhibitors have not been used in head and neck cancer chemotherapy, the higher expression of Topo IIα in HNSCC than in HNM appears to be linked to cell proliferation in tumor specimens, especially metastatic lymph node specimens, and therefore it may be worth considering the use of topoisomerase-targeting antitumor drugs against HNSCC.

We also found in this study that the *MDR1*, *MRP* and *LRP* genes were expressed in HNSCC and HNM to varying degrees. There have been several reports on the expression of these three proteins or genes in several normal tissues and cancer specimens.[7, 8, 25, 27–31, 36–38] However, there are only a few reports on the expression of P-gp, MRP and LRP in head and neck carcinomas using immunohistochemistry,[38–42] and the role of these genes in head and neck cancer or normal cells is unclear. The present report is the first to document the expression of the *MDR1*, *MRP*, *LRP* genes in HNSCC and HNM specimens based on molecular-biological methods. The results of various studies suggest that MDR-related genes have important roles, not only in the MDR phenomenon, but also in tumorigenesis and tumor progression. Schneider *et al*.[31] speculated that the genes associated with oncogenic development may also activate P-gp concomitantly in human cancers. Duensing *et al*.[43] showed that P-gp is a marker

for tumor progression in renal cell carcinomas. Therefore, we attempted to clarify the biological characteristics of the *MDR1*, *MRP* and *LRP* genes in HNSCC and HNM. There were no significant differences in these three genes between HNSCC and HNM, or between metastatic lymph node samples and HNM. Our results suggest that MDR1, MRP and LRP are not related to oncogenic activation in HNSCC.

Furthermore, previous reports demonstrated that MDR1 and MRP play important roles in resistance to Topo II inhibitors such as etoposide or adriamycin.[8, 22, 23] We did not detect any differences between the steady-state levels of MDR1 or MRP mRNA in HNSCC and HNM, suggesting that it may be worth considering the use of topoisomerase-targeting antitumor drugs against HNSCC.

Platinum drugs are frequently used in the chemotherapy of head and neck carcinomas. It has been suggested that MRP, cMOAT and LRP expressions correlate with resistance to platinum drugs.[5, 44, 45] Therefore, in this study we also examined the gene expression levels of these genes between samples exposed and not exposed to platinum drugs, and we found no differences among the steady-state levels of MRP and LRP mRNA expression levels between samples exposed to platinum drugs and those not exposed to platinum drugs; further, we did not detect *cMOAT* gene expression (data not shown). These results suggest that the expressions are not associated with platinum drug exposure in HNSCC, although the relationship between baseline expression levels and response to platinum drugs remains to be elucidated. We did not analyze factors such as thymidylate synthase[46] or dihydropyrimidine dehydrogenase[47] that are supposed to be associated with 5-fluorouracil (5-FU) sensitivity. Activities or expression levels of these enzymes may influence the effectiveness of 5-FU or the prognosis of HNSCC patients. Further studies will be required to analyze whether the activities or the expression levels of these genes are associated with 5-FU sensitivity before and after drug administration.

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