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Quantitative Analysis of Multidrug-resistance *mdr1* Gene Expression in Head and Neck Cancer by Real-time RT-PCR

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Progression of head and neck cancer is always associated with changes of gene expression profile. In this study, we characterized the expression of multidrug-resistance *mdr1* gene, which may play a role in tumorigenesis and multidrug resistance in head and neck cancer. A TaqMan one-step RT-PCR with a linear range for quantification across at least a 5 log scale of concentration of *mdr1* mRNA was designed to determine the level of *mdr1* expression in 50 pairs of normal vs. malignant head and neck tissues. Both the absolute level of *mdr1* mRNA in tumor (T) and the relative *mdr1* expression between tumor and its normal counterpart (T/N) were measured and their associations with several clinical variables were analyzed. Among the clinical variables analyzed, only the clinical stage of tumor was found to be associated with *mdr1* expression. The distribution of clinical stages differed significantly ($P<0.01$) among the 27 specimens that had a T/N>1, with 59.3%, 22.2%, 14.8% and 3.7% in stage IV, III, II, and I, respectively. In addition, 76% of stage IV and 75% of stage III tumors had a T/N>1 compared to 25% of stage II and 20% of stage I tumors ($P=0.004$). Multivariate logistic regression analysis also indicated a significant difference of *mdr1* expression between the early (I and II) and advanced (III and IV) stages tumors. The adjusted odds ratios (95% confidence intervals) were 1.477 (1.084–2.012) and 1.001 (1.000–1.002) for T/N ($P<0.05$) and T ($P<0.05$) treated as continuous variables, and 15.521 (3.414–70.550) and 5.074 (1.154–22.311) for T/N ($P<0.001$) and T ($P<0.05$) treated as binary variables, respectively. Taken together, the data presented here indicated that real-time RT-PCR provides a quantitative way to monitor *mdr1* gene expression. The differential expression of *mdr1* between early and advanced stages of head and neck cancer may shed light on the process of tumorigenicity and offer clues to the planning of new treatments.

Key words: Real-time RT-PCR — Multidrug resistant gene — Head and neck cancer

Head and neck cancer is the 6th most common cancer globally and the 5th major cause of cancer death in Taiwan. The progression of head and neck cancer is usually associated with changes of gene expression profile and involves multiple steps of gene activation and inactivation. In general, proto-oncogenes and protein kinases that participate in the mitogenic pathways are up-regulated, while tumor suppressors and differentiation-related molecules are inactivated or down-regulated. For instance, C-MYC and protein kinase ERK are expressed prominently in advanced stages of head and neck cancer with mutation or reduced expression of p53, p21, and p27.[1–5] The differential gene expression profile may influence the intrinsic characteristics of tumors, resulting in differences of clinical responses such as drug resistance and disease-free survival. Understanding the gene expression patterns should provide guidance on patient care and lead to better disease surveillance.

Multidrug-resistance gene *mdr1* encodes a 170 kDa transmembrane protein that serves as an adenosine triphosphate (ATP)-dependent efflux pump.[6] Physiologically, MDR1 may be involved in lipid translocation across the plasma membrane, and may interfere with caspase-dependent apoptosis, indicating that MDR1 has multiple functions in the control of cellular activities.[7–9] In tumors, overexpression of MDR1 reduces the accumulation of cytotoxic drugs inside the cells and results in the multidrug-resistance phenotype.[10–12] Studies have also indicated that MDR1 may play a role in head and neck cancer tumorigenesis. With quantitative flow-cytometric analysis using the MRK-16 monoclonal antibody, Jain *et al.* analyzed the oral cancer population in India and revealed a significant increase of MDR1 in untreated primary oral tumors and in dysplastic lesions as compared with normal oral tissues.[13] Ralhan *et al.* further provided clinical evi-

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dence for a significant association between MDR1 and p53 in head and neck cancer that may account for the aggressive nature of the tumor and the poor prognosis.[14] Rabkin *et al.* also found an association between higher MDR1 expression and decrease of survival rate.[15] Due to the lack of an appropriate and simple method for quantification of gene expression, there have been few studies to quantify *mdr1* mRNA expression in head and neck cancer. In addition, the distinct components of betel nuts consumed by the residents in Taiwan may lead to a different gene expression profile compared to those in other countries or regions. Thus, it is worthwhile to determine the levels of *mdr1* expression in head and neck cancer and to attempt to correlate its expression with clinical variables.

The recent advent of a real-time PCR technique has offered advantages for quantitative analysis of gene expression.[16, 17] Real-time PCR utilizes the 5′-nuclease activity of *Taq* DNA polymerase to cleave a nonextendable, fluorescence-labeled hybridization probe during the extension phase of PCR. The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethylrhodamine (TAMRA). The nuclease cleavage of the hybridization probe during PCR releases the effect of quenching, resulting in an increase of fluorescence proportional to the amount of PCR product. The fluorescence signal can be monitored by a sequence detector, such as the GenAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). In this study, we designed and performed a TaqMan probe-based, one-step real-time RT-PCR to determine the expression level of *mdr1* mRNA in 50 pairs of normal vs. malignant head and neck tissues. Our results indicate that *mdr1* is expressed differentially in different stages of head and neck cancer.

MATERIALS AND METHODS

Materials The T7MEGAshortscript *in vitro* transcription kit was purchased from Ambion (Austin, TX). The one-step TaqMan RT-PCR universal master mix reagent was purchased from Applied Biosystems (Foster City, CA). The Trizol reagent and the Superscript II reverse transcriptase were purchased from Life Technologies (Gaithersburg, MD). The TOPO TA cloning kit was purchased from Invitrogen (Groningen, The Netherlands).

Tissue specimens Biopsies, including 48 primary and 2 recurrent tumors, from 50 consecutive patients seen at the Otorhinolaryngology or Head and Neck Surgery Clinics at Chang Gung Memorial Hospital (Taoyuan, Taiwan) were obtained for study. Written informed consent was obtained from all patients participating in the study. Paired cancerous and normal mucosal tissue samples were obtained from each subject. A portion of each tissue sample was examined histopathologically, and the rest was stored in liquid nitrogen until use. All cancers were histologically graded as well differentiated, moderately differentiated, or poorly differentiated/undifferentiated according to the World Health Organization (WHO) classification.[18] Tumor stage and anatomic site were classified according to the AJCC system.[19] All patients received radical wide excision to remove the primary tumor with at least 1 cm margins and modified neck dissection depending on clinical neck lymph node metastasis status. Adjuvant radiotherapy was given to the patients with intermediate risk of recurrence. Adjuvant concomitant radiotherapy and chemotherapy were given to high-risk patients, such as those with multiple neck lymph node metastasis and/or extracapsular spreading of cancer. The chemotherapy agents were cisplatin combined with oral 5-fluorouracil and leucovorin.

Plasmid construction The *mdr1* cDNA fragment spanning nt 3384 to nt 3600 (GenBank Accession No. AF016535) was generated by RT-PCR with the primer set MDR1-F1 (5′-TTGGCAGGGAAAGTGCTGC-3′) and MDR1-R1 (5′-GCAGTGACTCGATGAAGGCA-3′). The PCR product was gel-purified and subcloned into the pTOPO-PCRII TA cloning vector to obtain pTOPO-MDR1. The orientation of the insert was confirmed by restriction mapping and sequencing.

***In vitro* transcription** The pTOPO-MDR1 (500 ng) was digested with *Hin*dIII and incubated at 37°C with 2 μl of 10× transcription buffer, 2 μl of ATP, 2 μl of CTP, 2 μl of GTP, 2 μl of UTP, and 2 μl of T7MEGAshortscript Enzyme Mix in a final volume of 20 μl. After reaction for 2 h, 1 μl of RNase-free DNase I mix was added to the reaction mixtures and incubated for an additional 15 min at 37°C. Then 15 μl of ammonium acetate precipitation solution was added and the RNA was ethanol-precipitated. The quality and quantity of cRNA were determined by agarose gel electrophoresis and ultraviolet spectroscopy.

Preparation of total RNA The total RNA was isolated with Trizol reagents as described by the manufacturer (Life Technologies). Briefly, the tissues were solubilized by homogenization in the Trizol reagents. One-tenth volume of chloroform was added to the sample. The tube was shaken vigorously, and kept on ice for 5 min. Following centrifugation (12 000*g*) at 4°C for 15 min, the RNA in the aqueous phase was transferred to a clean tube. The RNA was then precipitated with an equal volume of isopropanol and was redissolved in water with 1 μl of RNase inhibitor.

Real-time quantitative RT-PCR One-step real-time quantitative RT-PCR was performed with the GenAmp 5700 Sequence Detection System (Applied Biosystems). Briefly, 200 ng of total RNA was added to a reaction mixture containing 25 μl of 2× RT-PCR universal master mix, 2.5 μl of 40× reverse transcriptase mixture, 5 μl of forward primer MDR1-F2 (5 μM, 5′-CGTGTCCCAGGAGCCCATCCTG-3′), 5 μl of reverse primer MDR1-R2 (5 μM, 5′-CCACCCGGCTGTTGTCTCCA-3′), and 5 μl of TaqMan probe (2 μM, 6FAM-TGACTGCAGCATTGCT-

GAGAACATTGC-TAMRA) in a final volume of 50 μl. The conditions for RT-PCR were 1 cycle of 48°C for 30 min; 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold value (C_T) used to assess the quantity of target gene was determined as the point at which the fluorescence exceeded a preset limit. The copy numbers of *mdr1* mRNA in each sample were calculated based on the standard curve generated with the *in vitro*-transcribed *mdr1* cRNA. The data were then normalized by the expression of 18S RNA using TaqMan PDAR 18S RNA Control Reagent (Applied Biosystems) to control for the quality and quantity of the extracted RNA. The absolute level of *mdr1* mRNA in the tumor (T) and the relative *mdr1* expression between the tumor and its normal counterpart (T/N) were determined for statistical analysis.

Statistical analysis Statistical analysis was performed through either the χ^2 test or logistic regression analysis. Due to the small case numbers of stages I and III tumors, multivariate logistic regression analysis was performed with stages I and II treated as early stage, and stages III and IV as advanced stage. To estimate multivariate-adjusted odds ratios and their 95% confidence intervals for *mdr1* expression in early and advanced stages of head and neck cancer, risk factors for *mdr1* expression including age, sex, consumption of alcohol and betel, and smoking habit were included in the multiple logistic regression models. A P-value <0.05 was considered statistically significant.

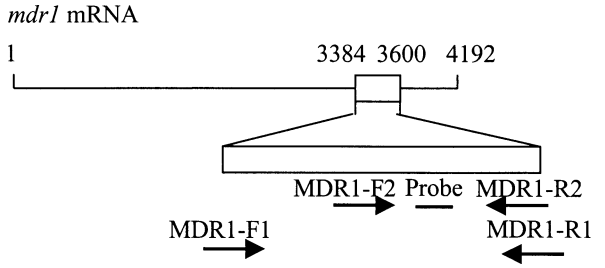


Fig. 1. Design of real-time RT-PCR for detection of *mdr1* mRNA. The relative positions of TaqMan probe and primers (MDR1-F2 and MDR1-R2) for real-time RT-PCR and for generation of *mdr1* cDNA (MDR1-F1 and MDR1-R1) are shown.

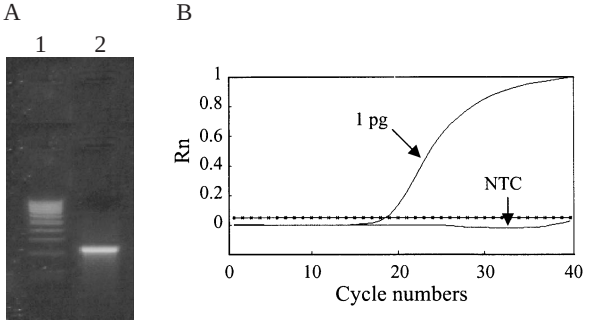


Fig. 2. Real-time RT-PCR for detection of *mdr1* mRNA. (A) The 217 bp *mdr1* cDNA fragment was subjected to *in vitro* transcription to produce the short transcript. The RNA product was fractionated on a 2% agarose gel followed by visualization with ethidium bromide staining. Lane 1, 100 bp markers; lane 2, *in vitro*-transcribed cRNA. (B) Typical amplification plot of real-time RT-PCR with 1 pg of *in vitro*-transcribed *mdr1* RNA as the template and the no template control (NTC). R_n, fluorescence signal.

RESULTS

The primers and TaqMan probe for detection of *mdr1* mRNA by one-step real-time RT-PCR were designed as shown in Fig. 1. The positive control cRNA (1 pg) generated by *in vitro* transcription of a 217 bp *mdr1* cDNA fragment was used as a template for the establishment of this technique (Fig. 2A). A typical amplification plot (change in fluorescent signal vs. cycle number) with a C_T of 18.12 was obtained (Fig. 2B). DNA sequence analysis of the PCR product confirmed the specific amplification of *mdr1* cRNA (data not shown).

The reproducibility and reliability of this assay were accessed by repeating the real-time RT-PCR four times under identical conditions (Table I). Each experiment was performed in quadruplicate. In the four experiments, the mean C_Ts were 18.33, 18.40, 18.57, and 18.64 and the intra-assay coefficients of variation (CVs) within each experiment (i.e. variations among the four sets of quadru-

Table I. Inter- and Intra-assay Variations of *mdr1* Real-time RT-PCR[a)]

Exp.	C_T	Mean	SD	CV (%)
1	18.12	18.33	0.32	1.7
	18.78			
	18.34			
	18.06			
2	17.94	18.40	0.51	2.7
	17.99			
	18.84			
	18.86			
3	18.66	18.57	0.10	0.5
	18.63			
	18.44			
	18.53			
4	18.30	18.64	0.25	1.3
	18.75			
	18.87			
	18.64			
Mean	—	18.94	0.14	0.7

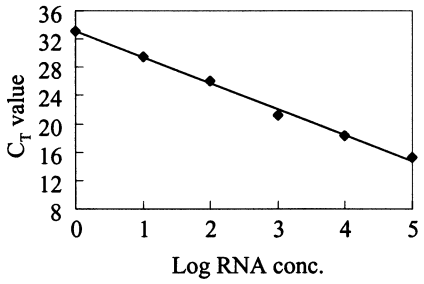
a) SD=standard deviation, CV=coefficient of variation.

plicates) were 1.7%, 2.7%, 0.5%, and 1.3%, respectively. Accordingly, the mean C_T was 18.49 [(18.33+18.40+18.57+18.64)/4], and the mean inter-assay CV was 0.7% [(1.7%+2.7%+0.5%+1.3%)/4].

To determine the detection limit of this method and to establish a standard curve that could be used for quantification, serial dilutions of *mdr1* control cRNA with a final concentration from 10 pg to 0.1 fg were subjected to real-time RT-PCR analysis (Fig. 3A). An amount as small as 0.1 fg of control RNA (C_T=33.14) in a 50 μl reaction volume could be detected (Fig. 3B). The standard curve was linear across at least a 5 log range of RNA concentration with a correlation coefficient of 0.9975 (Fig. 3A). Electrophoretic analysis of the real-time RT-PCR product on a 2% agarose gel showed the expected 217 bp band with decreasing intensity when the initial amount of the template decreased (data not shown).

Fifty pairs of malignant specimens and their normal counterparts from 48 males and 2 females with a mean age of 51.24 years were analyzed by *mdr1* real-time RT-PCR (Table II). The values for T and T/N, which represented the *mdr1* expression level as defined in "Materials and Methods," were determined for analysis of their association with clinical variables. The 50 tumor specimens indicated a trend of increase for *mdr1* expression in advanced stages (III and IV) of cancer (Fig. 4). Using T/N as the parameter (Table III), 54% of the patients (27/50) had a T/N>1. Among these 27 specimens, the distribution of the clinical stages differed significantly (P<0.01), with 59.3% (n=16), 22.2% (n=6), 14.8% (n=4) and 3.7% (n=1) in stages IV, III, II, and I, respectively. In addition, 76% (16/21) of the stage IV and 75% (6/8) of the stage III tumors overexpressed *mdr1* (i.e. T/N>1), which was significantly higher than 25% (4/16) of the stage II and 20% (1/5) of stage I tumors (P=0.004, Table III). With T/N and T as continuous or binary variables, the *mdr1* expression was significantly different between advanced stages (III and IV) and early stages (I and II) in multivariate logistic regression analyses (Table IV). All the other independent variables, including age, sex, and consumption habits for betel nut, alcohol, and tobacco, had no significant association with the expression of *mdr1* gene (data not shown).

A



B

C_T value	RNA conc.	Log value
15.29	10 pg	0
18.23	1 pg	1
21.22	100 fg	2
26.04	10 fg	3
29.55	1 fg	4
33.14	0.1 fg	5

Fig. 3. Establishment of the standard curve for quantification of *mdr1* mRNA. Ten-fold serial dilutions of *in vitro*-transcribed *mdr1* RNA, ranging from 10 pg to 0.1 fg, were used as the template for real-time RT-PCR assays. (A) C_T values were plotted against log [amount of RNA concentration]. (B) C_T values for all data points. Similar results were obtained in two independent experiments.

Table II. Characteristics of the Head and Neck Cancer Patients Enrolled in This Study

Sex	Male: 48 (96%)[a)]	
	Female: 2 (4%)	
Age	29–73 years old	
	Mean: 51.24	
Tumor sites	Oral cavity: 48 (96%)	Tongue: 17 (35.4%)
		Mouth floor: 3 (6.3%)
		Lip: 1 (2.1%)
		Buccal mucosa: 20 (41.6%)
		Alveolus: 6 (12.5%)
		Hard palate: 1 (2.1%)
	Oropharynx: 2 (4%)	

a) Except for age, the values represent the case numbers and the percentage in the respective category.

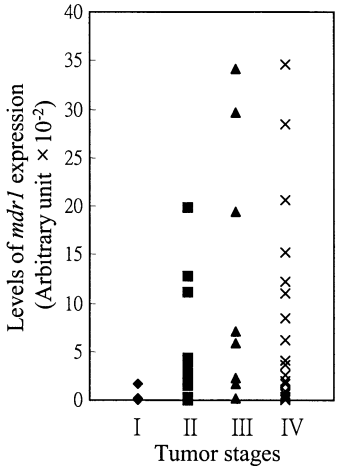


Fig. 4. Expression of *mdr1* mRNA in head and neck cancer. The absolute levels of *mdr1* mRNA in tumors (T) for 50 head and neck cancer specimens were plotted against the corresponding clinical stages.

Table III. Association of *mdr1* Expression and Clinical Stages of Tumors in Head and Neck Cancer[a)]

Tumor stages	*mdr1* expression (T/N)		*P*-value
	≤1	>1	
I (*n*=5)	4 (80%)[b)]	1 (20%)	
II (*n*=16)	12 (75%)	4 (25%)	
III (*n*=8)	2 (25%)	6 (75%)	0.004
IV (*n*=21)	5 (24%)	16 (76%)	
Total (*n*=50)	23 (46%)	27 (54%)	

a) χ^2 test; *P*-value <0.05 was considered to be significant.
b) The values represented the case numbers and the percentage in the indicated stage of tumor.

Table IV. Adjusted Odds Ratios for T/N and T Treated Either as Continuous or Binary Variables in Logistic Regression Analyses

Independent variables	Adjusted odds ratio (95% confidence interval)	*P*-value
Continuous		
T/N	1.477 (1.084–2.012)	<0.05
T	1.001 (1.000–1.002)	<0.05
Binary[a)]		
T/N	15.521 (3.414–70.550)	<0.001
T	5.074 (1.154–22.311)	<0.05

a) The categories were >1 vs. ≤1 for T/N, and >500 vs. ≤500 for T.

DISCUSSION

Taking advantage of its powerful quantification ability, a TaqMan one-step real-time RT-PCR was developed for specific and quantitative detection of *mdr1* mRNA. The *mdr1* real-time RT-PCR is extremely sensitive (0.1 fg of control RNA/reaction) and highly reproducible (mean inter-assay CV of 0.7%). With the ability to quantify the level of gene expression and its linear response over at least a 5 log range of RNA concentration (Fig. 3), this technique provides a new avenue for understanding the *mdr1* gene expression profile in head and neck cancer.

In this study, the expression of *mdr1* was analyzed by real-time RT-PCR with 50 tumor specimens in different clinical stages of head and neck cancer. The analysis focused on the mRNA, but not the protein, level, and thus eliminates the concern of the cross-reactivity of commonly used anti-MDR1 antibodies with several MDR1-related or non-related proteins such as MDR3 and tubulin.[20–23)] The data presented here suggest a significant association between *mdr1* and clinical stages of tumors, with up-regulation of *mdr1* mRNA in advanced stages (III and IV) head and neck cancer. These results are consistent with reports by others.[13–15)] The transactivation of *mdr1* seems not to be limited to head and neck cancer. Increased expression of the *mdr1* gene has been shown in experimental hepatocarcinogenesis and regenerating rat liver.[24)] Overexpression of P-glycoprotein has also been shown in adenomatous hyperplasia of human liver with cirrhosis[25)] and adenomas and adenocarcinomas of patients with familial adenomatous polyposis.[26)] In a variety of tumors such as colon and liver cancer, overexpression of *mdr1* gene results in multidrug resistance during the course of chemotherapy and may serve as a molecular marker for diagnosis of chemoresistance in clinical specimens.[10–12)] The increase of *mdr1* in advanced stages of head and neck cancer suggests that these tumors are likely to exhibit intrinsic drug resistance and may explain the limited usage of chemotherapy as the primary treatment in the management of head and neck cancer, particularly those arising in the oral cavity.

For untreated primary head and neck tumors that are the major subjects in this study, induction of *mdr1* could also be an inherent part of tumorigenesis. Multistep carcinogenesis involving multiple genetic events has been proposed for the development of head and neck cancer.[27)] These events can lead to increased cell proliferation, loss of cell adhesion, and the ability to invade local tissue and metastasize to distant sites.[28)] Previous studies indicated that the up-regulation of *mdr1* may, in addition to confirming the multidrug-resistance phenotype of malignant tissues, be involved in tumor progression from early stage to advanced stage or even to lymph node metastasis. Analysis of 95 primary colon adenocarcinomas by monoclonal antibody immunohistochemistry indicated that there was a significantly greater incidence of vessel invasion and lymph node metastasis in the subpopulation of invasive carcinoma cells that expressed P-glycoprotein.[29)] It was proposed that MDR1 may suppress cell death and lead to tumor progression by secreting endogenous or xenobiotic toxic substances or by mediating antiapoptotic effects through inhibition of the caspase-dependent cell death pathway.[9, 30)] A number of studies have also shown that increased P-glycoprotein expression is associated with changes of expression of a family of surface glycoproteins.[31–33)] Particularly, a progressive down-regulation of intercellular adhesion molecule-1 (ICAM-1), CD44, very late activation antigen (VLA)-5 and VLA-2 occurred in parallel with an increasing level of P-glycoprotein expression in melanoma cell line M14Dx.[34)] Based on these experimental findings, the association of *mdr1* overexpression in advanced stages of head and neck cancer is believed to play a role in the tumor aggressiveness and progression of the malignant tissues. Assessment of the disease-free survival in patients with different levels of *mdr1* will be essential to define the biological significance of *mdr1* overexpression in head and neck cancer.

At the molecular level, the tumor suppressor gene *p53* has been suggested to be involved in the up-regulation of *mdr1*. Recent work using PCR-single strand conformation polymorphism and DNA sequencing revealed that 48.66% of oral squamous cell carcinoma showed p53 mutations at conserved regions of the *p53* gene.[3] The study by Sampath *et al.* indicates that mutant p53 interacts and cooperates with ETS transcription factor in the up-regulation of human *mdr1* promoter.[35] Notably, immunohistochemical analysis of *p53* and *mdr1* gene product P-gp demonstrated a significant association between P-gp and mutant p53 protein expression in head and neck cancer.[14] The epidemiological factors that cause the mutation of p53 and possibly the induction of *mdr1* are not clear. It has been speculated that consumption of tobacco, alcohol, and betel nuts may play a role in the high incidence in several populations such as those of India and Taiwan.[36, 37] Patients with alcohol use, but not cigarette smoking or betel nut chewing, have been shown recently to have a significantly higher incidence of p53 mutations in oral squamous cell carcinomas.[3] The data from the present study do not support a role of any of these three factors in the induction of *mdr1*. This may due to either the small cases numbers used in the study, or the involvement of other epidemiological and molecular factors, in addition to p53 mutations, in the up-regulation of *mdr1*. A recent study revealed that, in addition to p53, the T-cell factor 4 (TCF4)/β-catenin complex is a transactivator of the *mdr1* gene in colorectal carcinogenesis.[26] These results, taken together, imply that multiple factors are involved in the induction of *mdr1* during the progression of malignant tissues, and may explain the late induction of *mdr1* in advanced stages of head and neck cancer.

In summary, the TaqMan real-time RT-PCR method described in this study provides a rapid, sensitive, and quantitative method for detection of *mdr1* mRNA in clinical specimens. The differential expression of *mdr1* between early and advanced stages of head and neck cancer may provide insight for understanding the process of tumorigenicity and for planning new treatment strategies.

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