Upregulation of thioredoxin reductase 1 in human oral squamous cell carcinoma

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Abstract. Thioredoxin reductase 1 (TrxR1) catalyzes the nicotinamide adenine dinucleotide phosphate-dependent reduction of oxidized thioredoxin (Trx). Trx, which is over-expressed in many human tumors, is a selenocysteine-containing protein associated with cell proliferation and apoptosis inhibition. This selenium-containing redox system regulates the activity of various enzymes and counteracts oxidative stress in cells such as hypoxia and cytotoxic agents. Consequently, TrxR1 could play an important role in tumor progression and resistance to chemotherapy due to its anti-apoptotic functions. To characterize cancer-related gene expression changes in oral squamous cell carcinomas (OSCC), we compared the gene expression profiles in OSCC primary tumors with patient-matched normal oral epithelium. Microarray analysis showed TrxR1 upregulation in primary tumors. Gene ontology analysis showed highly significant cancer-related function. The TrxR1 expression examined by immunohistochemistry was correlated with regional lymph node metastasis ($P<0.05$) and the clinical stages of 50 patients ($P<0.01$). Overexpression of TrxR1 could contribute to cancer progression and might be a potential molecular marker for therapy.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the leading malignancies and major causes of morbidity and mortality (1-3). Oral cancer is the most common cancer of HNSCC. The most frequent type of oral cancer is squamous cell carcinoma (OSCC) that accounts for more than 90% of all oral malignancies (4). The number of OSCC cases occurring worldwide annually exceeds 300,000 (1,5). With advances in surgical and radiation therapies in recent decades, patients diagnosed with stages I and II have a relatively good prognosis. However, patients with stages III and IV, which account for more than two-thirds of cases, have a high recurrence rate at regional and distal sites of metastasis (6,7). The resulting survival rates of all patients with OSCC remain poor. To improve the prognosis, strategies have been developed to integrate systemic chemotherapy into the perioperative period. Moreover, molecular targeted therapy recently has been extensively investigated as a single modality and in combination with cytotoxic treatments (8). Although these developments have significantly improved patient outcomes, the 5-year survival rates are less than 50% (9). In addition, the mechanisms behind tumor progression of OSCC are known to a limited extent, indicating a clear need for comprehensive knowledge leading to more specific and effective molecular target.

Microarray technology facilitates simultaneous evaluation of tens of thousands of genes within a specimen. The results of microarray analysis provide researchers with high-throughput screening to study the roles played by specific genes in cancer development and progression. Previous studies have identified a number of novel genes with altered expression in OSCC (10,11); however, these studies have not yet elucidated the role of these genes in a regulatory network of tumor progression. We performed microarray analysis using high-density Affymetrix Human Genome-U133A plus 2.0 GeneChip arrays containing 54,675 probe sets (Affymetrix, Santa Clara, CA) to compare gene expression patterns among OSCC primary tumors and patient-matched normal oral epithelium. We also adopted a computational tool, Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Mountain View, CA), to identify regulatory networks of differentially expressed genes related to OSCC.

In the current study, we analyzed the gene expression profiles of OSCC using microarray technology in combination with network and gene ontology by IPA to identify networks of interacting genes and other functional groups. We also verified experimentally that the selenocysteine-containing redox protein, thioredoxin reductase 1 (TrxR1), is upregulated in OSCC. In addition, we evaluated the correlation between TrxR1 expression and clinicopathological parameters to explore the therapeutic potential of TrxR1.

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Materials and methods

Tissue specimens and cell lines. Tumors and patient-matched normal epithelial specimens were obtained during surgical resection at Chiba University Hospital after the study patients provided informed consent for a protocol reviewed and approved by the institutional review board of Chiba University. The resected tissues were divided; one part was frozen immediately after removal of the surrounding normal tissues and stored at -80˚C until protein isolation, and the second part was fixed in 10% buffered formaldehyde solution for pathological diagnosis and immunohistochemical staining. The Department of Pathology, Chiba University Hospital, performed histopathological diagnosis of each neoplastic tissue according to the World Health Organization criteria. Clinicopathological staging was determined by the TNM classification of the International Union against Cancer. All patients had histologically confirmed SCC, and tumor samples were checked to ensure that tumor tissue was present in more than 80% of the specimens. The OSCC-derived cell lines used in this study were HSC-2, HSC-3, HSC-4, KON (Human Science Research Resources Bank, Osaka, Japan), H1, and Sa3 (provided by Dr Shigeyuki Fujita at Wakayama Medical University, Wakayama, Japan). All OSCC-derived cell lines were grown in Dulbecco's modified Eagle's medium/F-12 HAM (Sigma-Aldrich Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and 50 units/ml penicillin and streptomycin (Sigma). Healthy oral gingival specimens were collected from 27- to 86-year old patients at Chiba University Hospital. Five primary independent HNOKs were cultured and maintained in defined keratinocyte-SFM (Gibco-BRL, Gaithusberg, Germany) (12).

Affymetrix GeneChip hybridization. Double-stranded cDNA was synthesized from 20 μg of total RNA using the Superscript Choice system (Invitrogen Life Technologies, Carlsbad, CA, USA). After phenol/chloroform extraction and ethanol precipitation, a biotin-labeled *in vitro* transcription reaction was carried out using the cDNA template (Enzo Bioarray, Farmingdale, NY). cRNA (7 μg) was fragmented according to Affymetrix protocols and added to the recommended hybridization mixture. Expression profiles were created using the Human Genome U 133 Plus 2.0 arrays containing 54,675 probe sets (Affymetrix). Arrays were stained with phycoerythrin-streptavidin, and the signal intensity was amplified by treatment with a biotin-conjugated anti-streptavidin antibody followed by a second staining with phycoerythrin-streptavidin. Arrays stained a second time were scanned using the Affymetrix GeneChip Scanner 3000.

Data analysis. GeneChip analysis was performed based on the Affymetrix GeneChip Manual with Microarray Analysis Suite 5.0, Data Mining Tool 2.0, and Microarray Database software. All genes on the GeneChip were globally normalized and scaled to a signal intensity of 500. The Microarray Analysis Suite software used Wilcoxon's test to generate detected (present or absent) calls and used the calls to statistically determine if a transcript was expressed or not. After being filtered through a 'present' call ($P<0.05$), the expression data were analyzed using GeneChip Operating Software 1.1 (Affymetrix) and GeneSpring 6.1 (Silicon Genetics, Redwood City, CA). Fold changes were calculated by comparing transcripts between OSCC primary tumors with patient-matched normal oral epithelium.

mRNA extraction. mRNA was extracted when the cells reached 80-90% confluence; they were washed twice with phosphate-buffered saline (PBS), scraped into a tube, and centrifuged briefly. Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The extracted RNA samples were stored separately at -80˚C until use.

mRNA expression analysis. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to evaluate the expression level of TrxR1 mRNA in the OSCC-derived cell lines (HSC-2, HSC-3, HSC-4, KON, H1 and Sa3) and HNOKs. Primary tumors and paired specimens of normal oral tissues from 50 patients also were evaluated. qRT-PCR was performed with a single method using a LightCycler FastStart DNA Master SYBR-Green 1 Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the procedure provided by the manufacturer. The primer sequences used to analyze TrxR1 mRNA expression were forward: 5'-GTTGCCAAGACTGCAAACCAC-3' and reverse: 5'-CCCTGCCAAATGTCAGCTTC-3'. The sequence of specific primers was checked before use to avoid amplification of genomic DNA or pseudogenes by Primer3 program (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain size and purity. The PCR reactions using the LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR-Green I mix, 3 mM $MgCl_2$, and 0.2 μl of the primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 63˚C (10 sec) for annealing, and 72˚C for extension, with a temperature slope of 20˚C/sec, performed in the LightCycler. The transcript amount for the TrxR1 gene was estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-CATCTCTGCCCCCTC TGCTGA-3' and reverse 5'-GGATGACCTTGCCCACA GCCT-3') transcript amount determined in corresponding samples. The significance of the gene expression levels between TrxR1-positive and -negative cases was calculated using the Mann-Whitney's U test, with $P<0.05$ considered significant.

Immunohistochemistry. Immunohistochemical staining was performed on 4-μm sections of paraffin-embedded specimens using rabbit anti-TrxR1 polyclonal antibody (ProteinTech Group, Chicago, IL). Briefly, after deparaffinization and hydration, the slides were treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min, after which the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS

Table I. Clinicopathological characteristics of primary OSCC for microarray analysis (n=3).

Tumor no.	Sex	Age at surgery	Tumor stage	pN	Stage	Histopathological type	Tumor site
1	F	67	T4	pN0	IV	Moderate	Buccal mucosa
2	M	49	T3	pN0	III	Moderate	Tongue
3	F	68	T1	pN2	IV	Moderate	Tongue

Table II. Four networks formed by overexpressed genes in OSCC clinical samples.

Genetic components in ingenuity networks[a]	Functions	Score[b]
Ap1, *BCAN*, *COL10A1*, ***COL11A2***, *Collagen type I*, *Collagen(s)*, ***CYP27B1***, ***ETV5***, *F12*, *Fibrin*, *Fibrinogen*, ***FN1***, ***GAD1***, *HAPLN1*, *Histone h3*, ***HOXA1***, ***HOXA10***, ***ITGB6***, ***LAMA1***, ***LAMC2***, *Laminin*, *LRG1*, *Mmp*, ***MMP7***, ***MMP11***, ***MMP13***, *MMP19*, *MMP25*, *MMP26*, ***PLAU***, ***PML***, *RNA polymerase II*, *SPARC*, ***SPP1***, *TMPRSS6*	i) Post-translational modification ii) Connective tissue disorders ii) Genetic disorder	31
ACP5, *ARNT2*, ***CNGB1***, ***COL11A1***, ***COL12A1***, *CREB1*, ***DLGAP1***, *DLGAP2*, ***DLGAP3***, *EGR2*, *ETS1*, *ETS2*, *FASLG*, *FOSL1*, *GRIN2A*, ***GRP***, ***HOXC6***, ***IBSP***, *IL1*, *JUN*, *KLK2*, *LAMA2*, *LAMB3*, *LTF*, *MME*, ***MMP13***, *NFYA*, *NMBR*, ***PDE4A***, ***PTHLH***, *RUNX2*, *SRC*, *TNFSF11*, ***TREM2***, *TYROBP*	i) Cellular development ii) Skeletal and muscular system iii) Development and function iv) Connective tissue development and function	12
AHNAK, *C3*, *CAV1*, *DNAH1*, *DNAH2*, ***DNAH5***, *DNAH6*, *DNAH9*, *DNAH10, DNAH12, DNAH14, DNAH17, DNAI1, DNAI2, DNAL1*, ***GPC6***, ***GPR77***, ***GYPA***, *HNF4A*, ***INHBA***, *INHBC, INHBE, JUNB*, *MAPK1*, ***MSMB***, *PGM1*, ***PMEPA1***, ***POLR3A***, *POLR3F*, ***PTPRR***, *RUNX1*, ***S100B***, *TIMP3, TP53*, ***TXNRD1***	i) Genetic disorder ii) Respiratory disease iii) Cellular growth and proliferation	11
A2M, ***ADAM12***, *CAV1, Collagen(s), CREBBP*, ***DLEU2***, *E2F1, ETS2*, *F2*, *F2RL1*, *Fibrinogen*, ***FN1***, *GNB2L1*, ***HOXD10***, ***IGF2BP2***, *ITGA9*, *ITGB1, ITGB1BP3*, ***LRP12***, *Metalloprotease, MYC, peptidase, PLG*, ***PROC***, *PROS1, RARG, REN, SLC3A2*, ***SLC7A8***, *SPARC*, ***SPP1***, *STAT3*, *Thyroid hormone receptor*, *VTN, YY1*	i) Cell-to-cell signaling and interaction ii) Cellular assembly and organization iii) Tissue development	9

[a]Genes in bold print were identified by microarray analysis as expressed differentially >5-fold in OSCCs. Other genes were either not on the expression array or not significantly regulated. [b]A score >3 was considered significant.

before reacting with anti-TrxR1 antibody (1:50 dilution) at room temperature in a moist chamber overnight. Upon incubation with the primary antibody, the specimens were washed three times in PBS and treated with Envision reagent followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako Japan Inc., Kyoto, Japan). Finally, the slides were counterstained lightly with hematoxylin, dehydrated with ethanol, cleaned with xylene, and mounted. As a negative control, duplicate sections were immunostained without exposure to primary antibodies. To quantitate the state of TrxR1 protein expression, the mean percentage of positive tumor cells was determined in at least five random fields at x400 magnification in each section. The expression of TrxR1 was semiquantitatively evaluated by two independent pathologists who were blinded to the clinicopathological characteristics of the patients. Cells were considered to be positive if cytoplasmic staining was observed. The intensity of the TrxR1 immunoreaction was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The percentage of positive tumor cells and the staining intensity were then multiplied to produce a TrxR1 immunohistochemical staining score (13,14). Cases with a TrxR1 score >110.8 (the highest score for normal tissues) were classified into high expression group. Statistical significance was evaluated by the χ^2 test or Mann-Whitney U test. $P<0.05$ was considered significant.

Results

Upregulated gene expression in OSCC primary tumors. To identify differentially expressed genes between OSCC primary tumors with patient-matched normal oral epithelium, we performed global gene expression analysis using Affymetrix GeneChips containing >54,000 probe sets (~39,000 transcripts and ~33,000 annotated genes) with RNAs isolated from a small cohort (n=3) of human tumors and matched normal oral tissues (Table I). Expression data were analyzed using the GeneChip Operating Software 1.1 and GeneSpring 6.1.

Table III. Five most frequently observed molecular functions in the identified genes related to disease and disorders.

Molecular function	P-value	Gene
Connective tissue disorders	4.99E-07-1.20E-02	22
Genetic disorders	4.99E-07-1.24E-03	51
Cancer	6.49E-07-1.17E-02	26
Gastrointestional disorders	6.49E-07-8.30E-03	27
Inflammatory disease	1.05E-05-1.20E-02	29

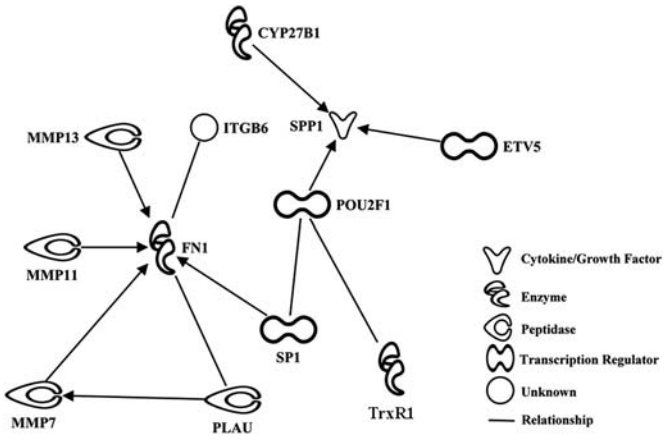

Figure 1. A network with 12 cancer-related genes upregulated in primary OSCC tumors compared with normal oral tissues. The functional relationship between gene products based on an known IPA knowledge base is described. The shapes of the nodes symbolize the functional class of gene product.

The results of microarray analysis showed that the expression levels of 131 genes were elevated at least 5-fold or more in all OSCC primary tumors compared with the matched normal tissues (data not shown).

Network and gene ontology analysis. Based on all genes identified as described previously (referred to as focus genes), new and expanded pathway maps and connections and specific gene-gene interactions were inferred, functionally analyzed, and used to build on the existing pathway using the IPA knowledge base. To generate networks, the knowledge base was queried for interactions between the focus genes and all other gene objects stored in the base. Four networks were significant in OSCC, in that they had more of the identified genes present than would be expected by chance (Table II). We also performed gene ontology analysis of 131 focus genes using the IPA tool. We were especially interested in identifying genes that had a relationship with cancer-related molecular function.

Twenty-six upregulated genes were associated with cancer-related molecular function (Table III). The cancer-related function was significant (P=6.49E-07-1.17E-02). Furthermore, to investigate the network of 26 cancer-related genes, we performed network analysis and identified one net-work (Fig. 1) that included 12 cancer-related genes (Table IV). Among the genes mapped to the cancer-related network, TrxR1 was analyzed further.

mRNA expression analysis. We examined the expression levels of TrxR1 mRNA in six OSCC-derived cell lines, HNOKs, primary tumors, and matched normal oral tissues from 50 patients with OSCC using qRT-PCR analysis. Significant upregulation of TrxR1 expression was observed in all OSCC cell lines examined compared with the HNOKs used as a control (Fig. 2A). Data are expressed as the means ± SD of two independent experiments with samples in triplicate. In addition, similar to the OSCC cell lines, the TrxR1 expression levels were upregulated in primary tumors compared with matched normal tissues (P<0.01) (Fig. 2B). qRT-PCR analysis showed upregulation of TrxR1 expression in 32 (64%) of 50 primary OSCCs compared with matched normal tissues. The relative mRNA expression levels, which were normalized to GAPDH, in the normal tissues and primary OSCCs ranged from 0.049

Table IV. Twelve cancer-related genes.

Affymetrix no.	Gene	Description	Fold change[a]
205959_at	*MMP13*	Matrix metalloproteinase 13	54.22
204259_at	*MMP7*	Matrix metalloproteinase 7	30.99
205676_at	*CYP27B1*	Cytochrome P450	30.02
209875_s_at	*SPP1*	Secreted phosphoprotein 1	26.20
203878_s_at	*MMP11*	Matrix metalloproteinase 11	14.19
205479_s_at	*PLAU*	Plasminogen activator	13.41
208083_s_at	*ITGB6*	Integrin, ß 6	11.06
210495_x_at	*FN1*	Fibronectin 1	7.33
201266_at	*TXNRD1*	Thioredoxin reductase 1	5.10
203349_s_at	*ETV5*	Ets variant gene 5	1.68
1553685_s_at	*SP1*	Sp1 transcription factor	1.57
206789_s_at	*POU2F1*	POU domain, class 2, transcription factor 1	1.03

[a]Fold overexpression for microarray data of primary tumors compared to normal epithelium. Underlining indicates genes identified by microarray analyses; other genes were not on the expression array.

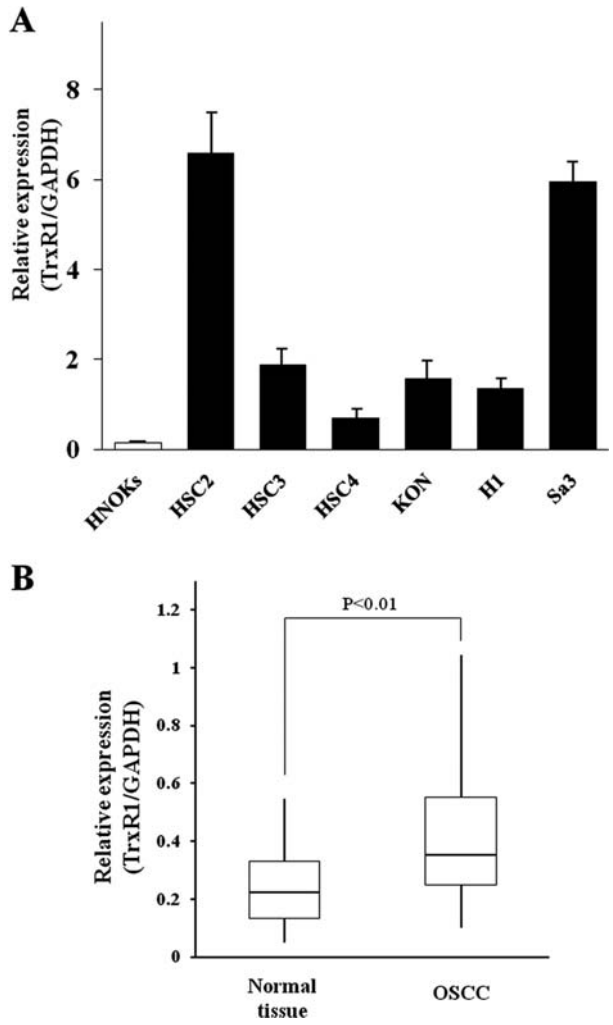

Figure 2. Increased TrxR1 mRNA expression in OSCC cell lines and primary OSCC tumors. (A) qRT-PCR analysis shows significant upregulation of TrxR1 mRNA expression in all six OSCC-derived cell lines compared with HNOKs. (B) The relative mRNA expression levels in normal tissues and primary OSCCs range from 0.049 to 0.55 (median, 0.22) and 0.10 to 1.04 (median, 0.36), respectively. qRT-PCR analysis shows significant (P<0.01) upregulation in primary OSCC tumors compared with matched normal tissues.

to 0.55 (median, 0.22) and 0.10 to 1.04 (median, 0.36), respectively.

Immunohistochemistry. Fifty patients with OSCC were identified for whom there was adequate histological material available for immunohistochemical analysis with corresponding normal tissues. Fig. 3 shows representative results for TrxR1 protein expression in normal oral tissue and primary OSCC tumors. The correlation between the clinicopathological characteristics of patients with OSCC and TrxR1 expression status is summarized in Table V. Among the tumors examined, 20 (40%) of 50 cases had high TrxR1 immunoreactivity. TrxR1 expression of these proteins was correlated with regional lymph node metastasis (P<0.05) and clinical stages (P<0.05, P<0.01, respectively). There were no significant correlations between TrxR1 expression and age, gender, histopathological type, or tumor site. The TrxR1 immunohistochemistry scores for normal tissues and primary OSCC

Table V. Correlation between TrxR1 expression and clinical classification in OSCC.

Clinical classification	Total	Results of immunostaining no. of patients (%) TrxR1 low	TrxR 1 high	P-value[a]
Age at surgery (years)				
<60	15	9 (60)	6 (40)	0.44
60-70	12	5 (42)	7 (58)	
>70	23	16 (70)	7 (30)	
Gender				
Male	32	17 (53)	15 (47)	0.19
Female	18	13 (72)	5 (28)	
T-primary tumor				
T1	8	7 (88)	1 (12)	0.11
T2	19	12 (63)	7 (37)	
T3	12	5 (42)	7 (58)	
T4	11	6 (55)	5 (45)	
N-regional lymph node				
N+	22	17 (77)	5 (23)	0.027
N-	28	13 (46)	15 (54)	
Stage				
I	7	6 (86)	1 (14)	0.007
II	12	9 (75)	3 (25)	
III	9	7 (78)	2 (22)	
IV	22	8 (36)	14 (64)	
I + II	19	15 (79)	4 (21)	0.032
III + IV	31	15 (48)	16 (52)	
Histopathological type				
Well	34	22 (65)	12 (35)	0.34
Moderately	14	8 (57)	6 (43)	
Poorly	2	0 (0)	2 (100)	
Tumor site				
Gingiva	8	5 (63)	3 (37)	0.57
Tongue	32	20 (63)	12 (37)	
Buccal mucosa	3	2 (67)	1 (33)	
Oral floor	6	3 (50)	3 (50)	
Soft palate	1	0 (0)	1 (100)	

[a]P<0.05 was considered significant.

ranged from 7.21 to 110.8 (median, 27.43) and 16.89 to 176.9 (median, 101.7), respectively. The TrxR1 expression levels in primary tumors were significantly (P<0.0001) higher than those in normal oral tissues (Fig. 4A). The TrxR1 immunohistochemistry scores for the OSCC group without regional lymph node metastasis and with metastasis ranged from 16.89 to 149.8 (median, 93.14) and 41.23 to 176.9 (median, 115.5), respectively. The TrxR1 expression levels were significantly (P<0.05) higher in the group with regional lymph node metastasis compared with the non-metastatic

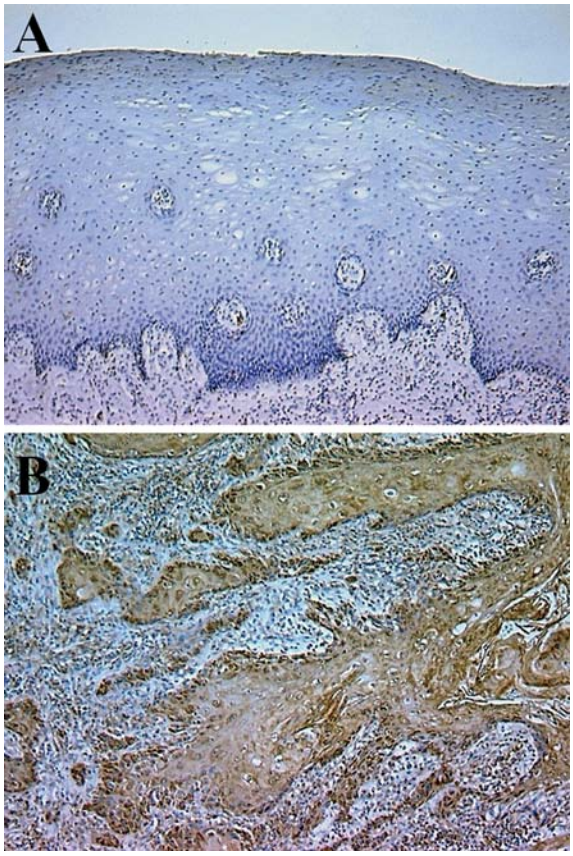

Figure 3. Immunohistochemical staining of TrxR1 in normal oral tissue and primary OSCC tumor. (A) A representative normal oral tissue has negative TrxR1 protein expression. (B) A representative primary OSCC tumor has positive TrxR1 protein expression with strong intensity in the cytoplasm.

group (Fig. 4B). Furthermore, the TrxR1 immunohistochemistry scores for the early stages (I and II) and advanced stages (III and IV) ranged from 16.89 to 149.8 (median, 92.81) and 41.23 to 176.9 (median, 117.1), respectively. The TrxR1 expression levels were significantly (P<0.001) higher in the OSCC group with advanced-stage disease, compared with the group with early-stage disease (Fig. 4C).

Discussion

Despite advances in surgery and radiotherapy during the past few decades, the survival rates of patients with HNSCC have improved only modestly. Moreover, OSCC is the most common form and has the third worst survival in HNSCC after hypopharynx and oropharynx (15). The mainstay of treatment of OSCC remains primary surgical resection (16). However, because the oral cavity regulates speech, deglutition, and mastication, aggressive surgical resection for patients with advanced disease can severely impair these functions and quality of life. Novel treatments are needed that can achieve organ preservation and to improve survival.

Microarray analysis revealed that 131 genes were upregulated in human OSCC primary tumors to matched normal oral tissues. Pathway analysis characterized four networks from the 131 genes (Table II). Gene ontology analysis also identified one network that included 12 cancer-related genes (Fig. 1). These investigations suggested that TrxR1 is upregulated in OSCC and plays an important role in tumor progression.

TrxR1 was named for its ability to reduce oxidized Trx, a group of small (10±12 kDa) ubiquitous redox-active peptides

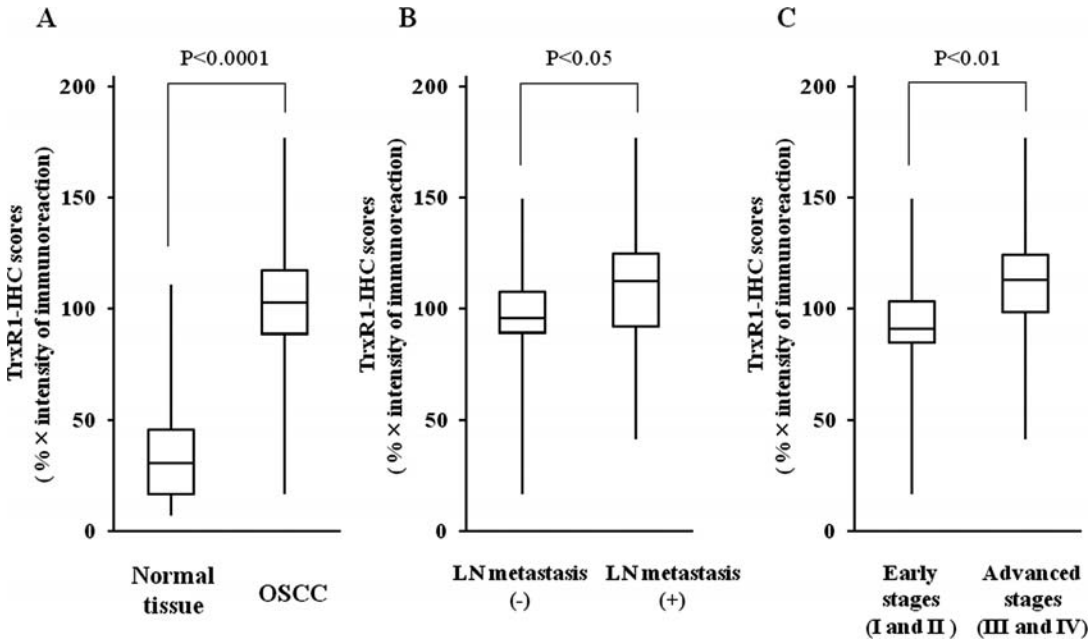

Figure 4. Immunohistochemical scores of TrxR1 expression in normal oral tissues and primary OSCC tumors. (A) Immunohistochemical scores for normal tissues and primary OSCC ranged from 7.21 to 110.8 (median, 27.43) and 16.89 to 176.9 (median, 101.7), respectively. The TrxR1 expression levels in primary tumors are significantly higher than in normal oral tissues (P<0.0001). (B) Immunohistochemical scores for the OSCC group without regional lymph node metastasis and with metastasis range from 16.89 to 149.8 (median, 93.14) and 41.23 to 176.9 (median, 115.5), respectively. The TrxR1 expression levels are significantly higher in the group with regional lymph node metastasis (P<0.05). (C) Immunohistochemical scores for the early-stages (I and II) and advanced stages (III and IV) range from 16.89 to 149.8 (median, 92.81) and 41.23 to 176.9 (median, 117.1), respectively. The TrxR1 expression levels are significantly higher in the OSCC group with advanced-stage disease (P<0.01). LN, lymph node.

with a conserved -Trp-Cys-Gly-Pro-Cys-Lys- catalytic site that undergoes reversible oxidation/reduction of the two Cys residues (17,18). Mammalian TrxR includes three isoforms, TrxR1 in the cytosol, TrxR2 in the mitochondria and TrxR3 primarily in the testes (19). Trx regulates the activity of various enzymes that counteract oxidative stress within cells. The reduced active form of Trx scavenging reactive oxygen species and induces the antiapoptotic signaling pathway. Inhibition of these Trx functions can disrupt the redox state and result in cell death (18,20). TrxR is the only enzyme in the cell that keeps Trx in the reduced state, and inhibition of TrxR leads to accumulation of oxidized Trx, resulting in cellular conditions that promote apoptosis (21). Consequently, this TrxR-Trx system plays an important role in cellular growth as a multifunctional active protein disulfide reductase system, widely distributed in nature, and can inhibit apoptosis by maintaining the redox state of various transcription factors including p53, ASK1, and NF-κB (18,21). Moreover, this redox system is associated with tumor angiogenesis by induction of vascular endothelial growth factor (20).

Cancer cells exist in a severely stressed environment and proliferate by angiogenesis and protection against various apoptotic signaling. The TrxR-Trx system is thought to have key functions in cancer cell proliferation. A number of reports have shown that the Trx-TrxR system has an important role in tumor growth and resistance to chemotherapeutic agents in many malignant neoplasms (22), i.e., prostate (23), pancreas (24), breast (25), non-small cell lung cancers (26), and malignant mesothelioma (27). Previous studies also reported that TrxR1 was one of the genes strongly associated with tumor proliferation in some cancers (23,28). Based on these data, the effect of TrxR inhibitors resveratrol and motexafin gadolinium have been investigated in cancer therapy (22,29,30).

Despite progress in other types of cancers, the state of this redox system in OSCC remains unclear. To overcome this, we selected TrxR1 from among the 12 cancer-related genes and investigated the expression of TrxR1 in cell lines and clinical specimens derived from OSCC. In qRT-PCR examination, significant increases in TrxR1 mRNA were seen in all of the examined OSCC-derived cell lines compared to HNOKs. We also detected tumor cell-localized cytoplasmic TrxR1 immunoreactivity in OSCC resected specimens. The expression levels of TrxR1 evaluated by immunohistochemistry scores were significantly overexpressed in primary OSCCs ($P<0.01$, OSCC vs. corresponding normal tissues). Immunohistochemical analysis of primary OSCC showed that the high TrxR1 expression group had a significant positive correlation with regional lymph node metastasis ($P=0.027$) and advanced pathological stages ($P=0.007$). Moreover, immunohistochemistry scores for the OSCC group with regional lymph node metastasis were significantly ($P<0.05$) higher than the group without regional lymph node metastasis. Previous studies reported that positive lymph node involvement was the most decisive factor for the prognosis of OSCC (31). In addition, immunohistochemistry scores for the group with stages III and IV disease were significantly higher than the group with stages I and II disease ($P<0.01$). These results suggested that upregulation of TrxR1 in OSCC may increase tumor aggressiveness and lead to poor prognosis.

In summary, the current study identified a new network of cancer-related genes, including TrxR1, in OSCC. We also showed that upregulation of TrxR1 is significantly associated with regional lymph node metastasis and the clinical disease stages. These results suggested that TrxR1 plays an important role in tumor progression and may be a potential prognostic marker and a new therapeutic target in OSCC.

Acknowledgments

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