Use of Real-Time Quantitative PCR to Identify High Expressed Genes in Head and Neck Squamous Cell Carcinoma Cell Lines

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Abstract

Head and neck squamous cell carcinoma(HNSCC) is the sixth most common cancer among men in the developed world affecting the tongue, pharynx, larynx and oral cavity. HNSCC is thought to represent a multistep process whereby carcinogen exposure leads to genetic instability in the tissue and accumulation of specific genetic events, which result in dysregulation of proliferation, differentiation, and cell loss and the acquisition of invasive capacity. Despite therapeutic and diagnostic progress in oncology during the past decades, the prognosis of HNSCC remains poor. Thus it seems that finding a biological tumor markers which will increase the early diagnosis and treatment monitoring rates, is of paramount importance in respect to improving prognosis.

In an effort to identify gene expression signatures that may serve as biomarkers, this study several genes were selected, such as H3,3A, S100A7, UCHL1, GSTP1, PAI-2, PLK, TGFβ1 and bFGF, and used 7 HNSCC cell lines that were established various anatomical sites, and also 17 other cancer cell lines were used for control group using real-time quantitative RT-PCR and immunocytochemical analysis with a monoclonal antibody.

In this study, S100A7 showed a clearly restricted occurrence in tongue originated cell line, and GSTP1 expression level in the pharynx originated cell line was very increased, relative to corresponding other cell lines. These results suggest that S100A7 and GSTP1 genes' expression can occur during tongue and pharynx originated head and neck tumorigenesis and that genetic change is an important driving force in the carcinogenesis process. This data indicate that S100A7 and GSTP1 expression pattern in HNSCC reflect both diagnostic clue and biological marker. And this is provides a foundation for the development of site-specific diagnostic strategies and treatments for HNSCC.

Key words

Real-Time Quantitative PCR, HNSCC, S100A7, GSTP1, Immunocytochemical analysis

INTRODUCTION

Head and neck squamous cell carcinoma(HNSCC) are neoplastic lesions found predominantly in the oral cavity, including the salivary glands, larynx and pharynx[1]. It is the sixth most common cancer and with an estimated annual worldwide incidence of 500,000 cases[2]. Despite significant advances in treatment, the 5-year survival rate of HNSCC is relatively lower than that of colorectal, cervix and breast cancer[3]. The high morbidity rate for this malignancy can be attributed to several factors, which include lack of suitable markers for early detection, late presentation, insensitivity to available treatment and our limited understanding of the molecular mechanisms responsible for this malignancy[4]. In this regard, the identity of those genes that may have a role in the progression of HNSCC has yet to be fully elucidated.

The National Cancer Institute(NCI) established The Cancer Genome Anatomy Project(CGAP), whose goal has been to compile a complete catalogue of all genes expressed in different tumor cells. CGAP' s sixth focus is about HNSCC[5]. CGAP generated sequence information

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initially from representational cDNA libraries derived from HNSCC cell lines and primary oral keratinocytes, to gain information on the nature of those genes expressed in this particular cell type[6]. Among those genes, several candidate genes were selected for this study. The genes were H3 histone family S100A7, 3A(H3.3A), ubiquitin C-terminal hydrolase L1(UCHL1), glutathion S-transferase P1(GSTP1), plasminogen activator inhibitor-2(PAI-2), polo-like kinase(PLK), transforming growth factorβ1(TGFβ1), and basic fibroblast growth factor(bFGF). These are thought to be positively related to HNSCC development.

This study applied gene expression through the Real-Time Quantitative PCR (RTQ-PCR) and immunocytochemical method to a group of 7 HNSCC cell lines and compared the genes expression to 6 gastric cancer cell lines, 5 lung cancer cell lines, 2 prostate cancer cell lines, 2 colon cancer cell lines, 1 kidney cancer cell line and 1 liver cancer cell line. This study's aims are to obtain a comprehensive view of gene expression differences in HNSCC cell lines and other carcinoma cell lines and to identify gene expression and localization signatures that correlate with defined clinical parameters with the hope of identifying the unique prognostic gene expression-based biomarkers in HNSCC.

MATERIALS AND METHODS

Cell lines and cell culture

Human head and neck squamous cancer cell lines (SCC-15, SCC-25, FaDu, RPMI 2650, CAL 27, SNU-1066 and SNU-1214), human gastric cancer cell lines (SNU-5, SNU-216, SNU-484, SNU-668, SNU-719 and MKN-74), human colon cancer cell lines (HT-29, HCT 116), human lung cancer cell lines (A549, H157, H358, H1299, H1703), human kidney cancer cell line (ACHN), human prostate cancer cell lines (DU 145, PC-3), and human liver cancer cell line (HepG2) were obtained from Korean Cell Line Bank (KCLB) and American Type Culture Collection (ATCC) (Table 1).

SNU-5, SNU-216, SNU-484, SNU-668, SNU-719, SNU-1066, SNU-1214, A549, H157, H358, H1299, H1703, MKN74 and PC3 were grown in RPMI1640 medium (Gibco/BRL), SCC-15 and SCC-25 were grown in DMEM/F-12 medium (Gibco/BRL), FaDu, ACHN, DU145, HepG2 and RPMI2650 were grown in MEM medium (Gibco/BRL), CAL27 was grown in DMEM medium (Gibco/BRL), HT-29 and HCT 116 were grown in McCoy's 5a medium (Gibco/BRL) in a humidified atmosphere containing 5% CO_2 at 37℃. All of these mediums were supplemented with 10% of heat-inactivated fetal bovine serum.

RNA extraction and cDNA preparation

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacture's instructions. RNA was resuspended in diethylpyrocarbonate-treated water and was quantitated by spectrophotometer. Purified 5 μg RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (MBI Fermentas) using Oligo(dT)18 primers and M-MuLV reverse transcriptase in a 20 μl reaction volume.

Table 1. Primary sites and tumor sources of cell lines

Cell lines	Primary site	Gen/Age, Race
SCC-15	tongue	M/55
SCC-25	tongue	M/70
FaDu	pharynx	M/56, Caucasian
CAL 27	tongue	M/56, Caucasian
RPMI 2650	nasal septum	M/52
SNU-1066	larynx, glottic	M/62, Mongoloid
SNU-1214	larynx, glottic	M/55, Mongoloid
SNU-5	stomach	F/33, Mongoloid
SNU-216	stomach	F/46, Mongoloid
SNU-484	stomach	M/53, Mongoloid
SNU-668	stomach	M/63, Mongoloid
SNU-719	stomach	M/53, Mongoloid
MKN-74	stomach	M/37, Mongoloid
A549	lung	M/58, Caucasian
H157	lung	
H358	lung	
H1299	lung	
H1703	lung	
HT-29	colon	F/44, Caucasian
HCT 116	colon	M
ACHN	kidney	M/22, Caucasian
DU 145	prostate	M/69, Caucasian
PC-3	prostate	M/62, Caucasian
HepG2	liver	

Primer design

The PCR primers were designed with the use of Primer Express 1.5 (Applied Biosystems). Each gene sequence was designed to produce approximately 100-bp amplicon. Primer sequences for amplification of S100A7, GSTP1, H3.3A, UCHL1, PAI-2, PLK, TGFβ1, bFGF and GAPDH by RTQ-PCR were listed in Table 2.

Real-Time Quantitative PCR (RTQ-PCR)

RTQ-PCR assays were carried out by using ABI Prism Sequence Detection System 7000 (PE Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems, warrington, UK). RTQ-PCR amplification was performed in 20 μl of reaction mixture containing 5 μl of cDNA samples, 10 μl of SYBR Green PCR Master Mix and 200 nM specific primer sets. cDNA samples and primers were diluted 10 fold. The thermal cycling conditions comprised two initial denaturation steps at 50℃ for 2 min and 95℃ for 10 min and 50 cycles at 95℃ for 15 sec and 60℃ for 1 min. All samples were measured in triplicate. A nontemplate control was included in each experiment. To ensure that amplifiable material was present in all of the cell lines and to avoid false-negative results, real-time amplification of housekeeping gene GAPDH was per-

formed for all of the samples. Dissociation curve analysis was performed at the end of 50 cycles to verify PCR product identity. The cycle threshold values of all samples were measured by the same machine. The final PCR products were run on 2% minigel to ensure single product amplification during the PCR assay.

Relative quantitation using the comparative Ct method

The comparative Ct method is similar to the standard curve method, except it uses arithmetic formulas to achieve the same result for relative quantitation. Quantitative values were obtained from the cycle number (Ct value) at which the increase in fluorescent signal associated with exponential growth of the PCR products. GAPDH was chosen for the housekeeping gene. Expressed as N-fold differences in target gene expression relative to the GAPDH gene, termed 'N$_{target}$', were determined by the formula N$_{target}$ = $2^{-\triangle\triangle Ct\ sample}$, where the $\triangle$Ct value of the sample was determined by subtracting the Ct value of the target gene from the average Ct value of the GAPDH gene. The calculation of $\triangle\triangle$Ct involves subtraction by the $\triangle$Ct calibrator value. The N$_{target}$ values of the samples were subsequently normalized such that the mean of the N$_{target}$ values of the control populations equal 1.

Table 2. Primers for RTQ-PCR

Gene	Accession No.	Primer (5'-3')	Annealing Temp.
S100A7	NM 002963	GATTGACAAGCCAAGCCTGC CAAAGACGTCGGCGAGGTAA	80℃
GSTP1	NM 000852	CAGGAGGGCTCACTCAAAGC AGGTGACGCAGGATGGTATTG	84℃
H3 3A	NM 002107	TGGTGCGAGAAATTGCTCAG AAGGCCAACCAGATAGGCCT	81℃
UCHL1	NM 004181	GAAGGCCAATGTCGGGTAGA GGAAAAGGCATTCGTCCATC	77℃
PAI-2	NM 021696	GTTACCCCCATGACTCCAGA CGCAGACTTCTCACCAAACA	81℃
PLK	NM005030	CGACTTCGTGTTCGTGGTGT GGTAGTATCGGGCCTCAGGC	85℃
TGFβ1	NM 000660	GCGTGCTAATGGTGGAAACC CGCTTCTCGGAGCTCTGATG	78℃
bFGF	NM002006	AAGCAGGAGGATCGCTTGAG TGTACACGCCACCATGCCT	80℃
GAPDH	NM002046	TGGGCTACACTGAGCACCAG GGGTGTCGCTGTTGAAGTCA	80℃

Immunocytochemistry

Cells were fixed for 20 min at room temperature in 2% Bax and permeabilized in 0.1% Triton-100 for 2 min. After a rinse in phosphate-buffered saline (PBS), cells were incubated for 1 hr with primary S100A7 monoclonal antibody (1:100 dilution, imgenex). After rinsing 3x5 min in PBS, cells were incubated with the secondary antibody (1:150 dilution, goat anti-mouse), for 40 min at room temperature and countstaining with SYTOX green, followed by dehydration, clearing and mounting. Stained cells were examined under a Nikon-Diaphot inverted microscope.

Statistical analysis

All determinations were made in triplicate, and the results were expressed as the mean standard deviation (S.D.). Statistical significance was determined by ANalysis Of VAriance (ANOVA) Duncan test. P values of 0.05 or less were considered significant.

RESULT

Relative quantitation of gene expression

Analysis of the gene expression patterns of HNSCC cell lines and other tumor cell lines has revealed S100A7 and

Table 3. Relative quantitation in S100A7 mRNA using the comparative($\triangle\triangle$ Ct) method

Cell line	$\triangle$Ct S100A7-GAPDH[a]	$\triangle\triangle$Ct $\triangle$Ct-$\triangle$Ct, SNU668[b]	S100A7 Rel.to SNU668[c]
SCC15	2.16	20.3	1290948
SCC25	6	16.46	90148
FaDu	10.21	12.25	4871
RPMI2650	17.33	5.13	35
CAL27	1.72	20.24	1751306
SNU1066	7.74	14.72	26987
SNU1214	6.83	15.63	50711
SNU5	17.87	4.59	24
SNU216	14.8	7.66	202
SNU668	22.46	0.0	1
MKN74	13.76	8.7	416
SNU484	17.87	4.59	24
SNU719	13.63	8.83	455
A549	14.11	8.35	326
H157	15.05	7.41	170
H358	13.13	9.33	644
H1299	18.95	3.51	11
H1703	22.33	0.13	1
DU145	12.6	9.86	929
PC3	14.29	8.17	288
HT29	15.8	6.66	101
HCT116	15.39	7.07	134
ACHN	14.16	8.3	315
HepG2	20.46	2	4

a. The $\triangle$Ct value is determined by subtracting the average GAPDH Ct value from the average S100A7 Ct value. The standard deviation of the difference is calculated from the standard deviations of the S100A7 and GAPDH values.

b. The calculation of $\triangle\triangle$Ct involves subtraction by the $\triangle$Ct calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of $\triangle\triangle$Ct is the same as the standard deviation of the $\triangle$Ct value.

c. The range given for S100A7N relative to SNU668 is determined by evaluating the expression $2^{-\triangle\triangle Ct}$ with $\triangle\triangle$Ct+s and $\triangle\triangle$Ct-s, where s = the standard deviation of the $\triangle\triangle$Ct value.
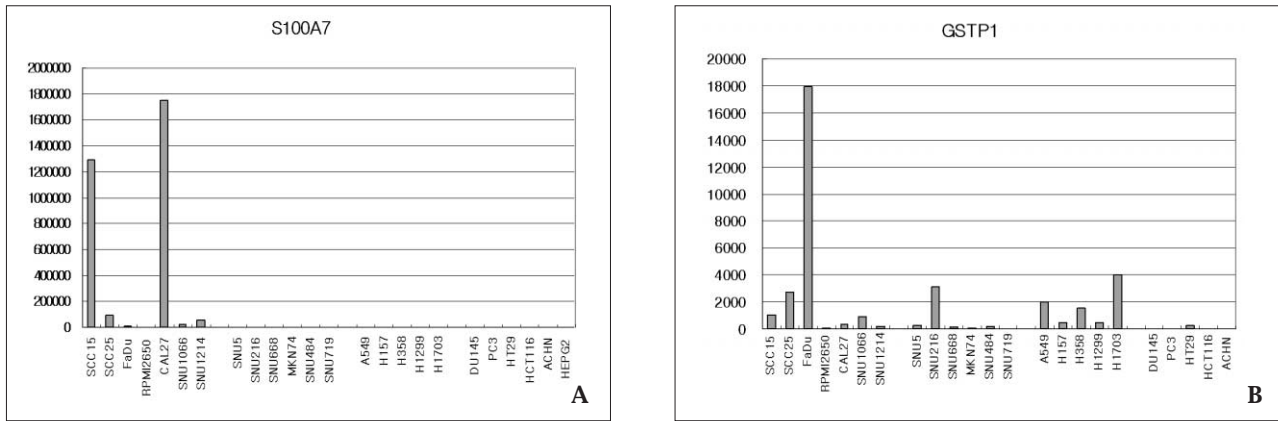
Fig. 1. Real-time quantitative RT-PCR analysis.

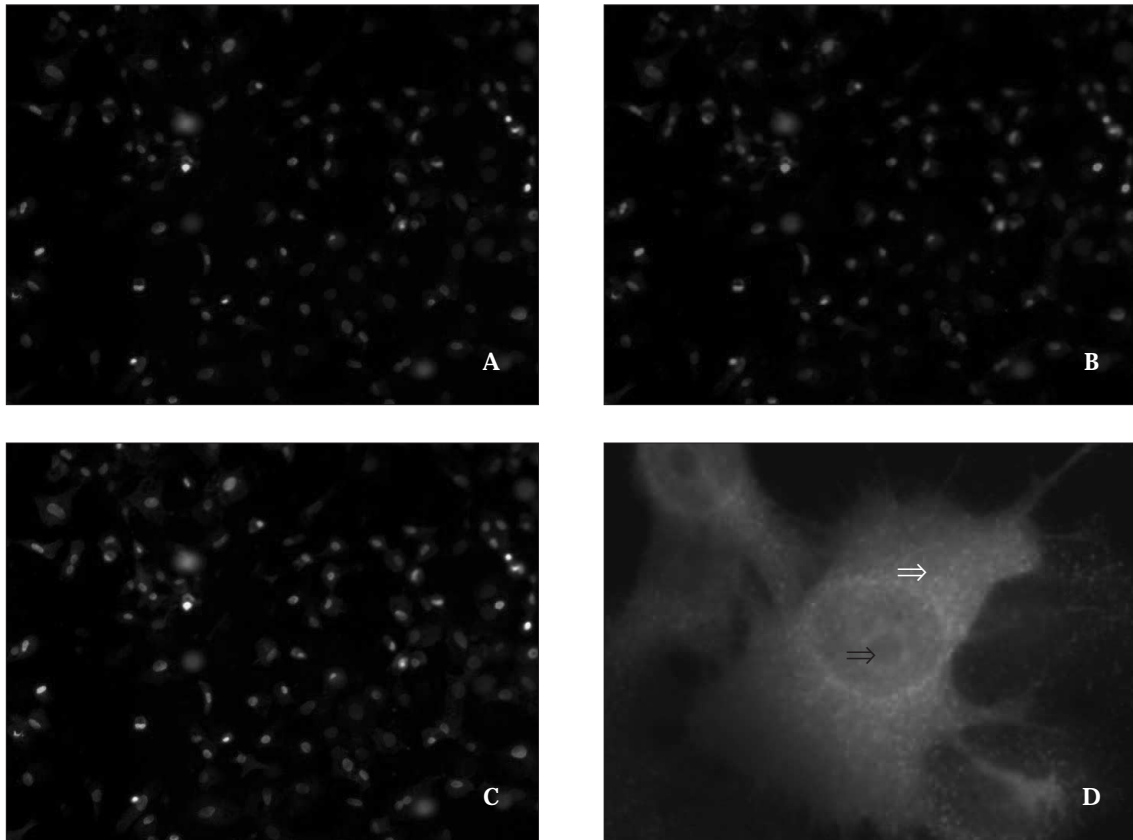**Fig. 2.** Immunocytochemical analysis of the cellular distribution and patterns of expression of S100A7 antibody within CAL27(A, B and C). ×200 and ×1000. S100A7 antibody is localized in CAL27 cell in cytoplasm(D, white arrow) and in nuclei(D, black arrow).

GSTP1 genes those are highly and specifically up-regulated (Table 3, only S100A7 gene's mRNA expression level was showed).

The tongue originated cell lines, such as SCC15, SCC25 and CAL27, expressed significantly high levels (the range given for S100A7 relative to SNU668 is determined by evaluating the expression $2^{-\triangle\triangle Ct}$), the highest S100A7 mRNA expression was observed in CAL27, while pharynx, nasal or larynx originated cell lines, such as FaDu, RPMI2650, SNU1066 and SNU1214, showed low levels.

In the other cell lines, this gene's mRNA expression was significantly low (Fig. 1A). In case of GSTP1 gene, the expression level was also high in HNSCC cell lines as S100A7 gene. FaDu cell line showed tremendous expression level of GSTP1 gene relative to ACHN. The expression of GSTP1 gene in other HNSCC cell lines and the other cell lines were significantly low (Fig. 1B).

H3.3A mRNA was expressed in the most of the HNSCC cell lines and the control group. UCHL1 mRNA showed its expression highly in lung cancer cell lines, the other cancer cell lines include HNSCC cell lines, showed very low levels. In case of UCHL1 gene, this study did not find some correlation with HNSCC cell lines. In case of PLK gene, similar pattern of expression was observed in all cancer cell lines, except HepG2 cell line, which showed high expression level. PAI-2 mRNA was expressed very low level in HNSCC cell lines and other cancer cell lines. TGFβ and bFGF gene were expressed very low level in the most cancer cell lines, except stomach cancer cell lines. The expression of these genes were not founded specificity with HNSCC cell lines(these genes' figures were not showed).

Immunocytochemistry

Because S100A7 mRNA expression was found to be higher than that in the other cancer cell lines, immunocytochemical analysis was carried for S100A7 first. S100A7 antibody was used in CAL27, SNU484, SNU1066, A549 and ACHN, and was expressed in CAL27 cells but in other cancer cells showed absent or barely detectable levels. Figure 2 showed CAL27 immunoactivity to S100A7 antibody. S100A7 monoclonal antibody was localized in cytoplasm (Fig. 2D, white arrow) and nucleus (Fig. 2D, black arrow). Original magnification for panels at the microscope, ×200 and ×1000.

DISCUSSION

The aim of this study was to identify the useful candidate genes related to HNSCC oncogenesis. In this study, the gene expression level was determined using a quantitative method, RTQ-PCR, which is considered to be a very accurate and reproducible method for assessing the gene expression level[7]. Among HNSCC-related genes[6], genes whose expression level has association with HNSCC development were selected and assessed their expression level in HNSCC cell lines. We found that

S100A7 gene and GSTP1 gene level in the HNSCC cell lines were significantly increased, relative to corresponding other cancer cell lines.

S100A7 gene was first detected as a low molecular mass calcium-binding protein in psoriatic keratinocytes[8-10]. This gene's postulated function is association with keratinocyte differentiation, psoriasis and breath and bladder cancer progression. A possible role for S100A7 in HNSCC is only emerged by Banerjee[11] as a invasion related biomarker and the exact function is not known. In our results, S100A7 gene expression showed a clearly restricted occurrence in tongue originated cell lines(CAL27, SCC15, and SCC25, especially increased in CAL27) and rarely detected in pharynx, larynx or nasal septum originated cell lines. In addition, to analyze the expression level and location of the S100A7 protein, this study performed immunocytochemical analysis of HNSCC cells using monoclonal anti-S100A7/HID-5 antibody. Figure 2 shows that S100A7 proteins localize in the cytoplasm and nucleus, this means that S100A7 protein performs function such as cell cycle progression and differentiation at both site. In HNSCC cell lines, the high expression of S100A7 gene suggests that it may play a role in the regulation of the HNSCC growth, survival or differentiation, so S100A7 gene could be one of the candidate for diagnostic marker in the tongue originated HNSCC.

The considerable importance of lifestyle in HNSCC, particularly the use of tobacco and alcohol and the role of diet, are well recognized[12]. However, despite the importance of these casual habits, relatively few persons who have them actually develope cancer, often despite years of exposure and, conversely, there are patients who develope HNSCC in no histology such habits or other identifiable lifestyle or environmental aetiological factors. Host susceptibility must therefore play a important role. Factors determining the individual susceptibility to HNSCC are still largely unknown. An imbalance between enzymes involved in the toxification and detoxification of (pre)-carcinogens closely related to HNSCC. Genetic polymorphisms in Glutathione S-transferases (GSTs) often result in altered detoxification, which may contribute to individual susceptibility to HNSCC[13]. GSTs was a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Based on their biochemical, immunologic, and structural properties, the soluble GSTs is categorized

into 5 main class : alpha, mu, pi, theta and zeta. The glutathione S-transferase pi gene (GSTP1) is a polymorphic gene encording active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases[14]. GSTP1 expression level, like S100A7 gene pattern, differs depending on the cell line. In HNSCC cell lines, GSTP1 showed a distinctly restricted occurrence in pharynx originated cell line (FaDu) and rarely detected in tongue, larynx, nasal septum originated cell lines and the other cancer cell lines. This results support the view that GSTP1 expression related to susceptibility to cancer of the pharynx. This data suggests that the patient with highly expressed GSTP1 pattern may be at increased risk of HNSCC of the pharynx.

CONCLUSION

These results are provides a foundation for the development of site-specific diagnostic strategies and treatments for HNSCC and also additional analysis are needed to elucidate the exact role of S100A7 and GSTP1 genes in HNSCC tumorigenesis.

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