Gene expression profile of oral squamous cell carcinomas from Sri Lankan betel quid users

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Abstract. Oral squamous cell carcinoma (OSCC) is one of the major health problems in Sri Lanka and the disease is associated with the habit of Betel Quid (BQ) chewing. Using 35k oligo microarrays, we analyzed the gene expression profile of 15 Sri Lankan patients diagnosed with OSCCs and pair-wised normal controls and correlated the findings with the clinicopathological data. Following the recording of the scanned array images and data analysis, results for selected candidate genes were verified using QRT-PCR. Upon analysis, a total of 263 genes [71 (27%) of unknown functions previously not reported in OSCCs and 192 (73%) of known functions] were found as differentially expressed between tumors and controls. For the genes with known functions, 66 (34%; such as *COL4A1*, *MMP1*, *MMP3*, *PLAU*, *SPARC* and *KRT19*) were previously reported in OSCC and for the remaining 126 (66%; such as *CD47*, *APOL3*, *RRAGC*, *BPIL1* and *AZGP1*) this is the first report in OSCCs. Hierarchical clustering of the differentially expressed 263 genes grouped the samples into several clusters with the larger one being dominated by tumors of stage 3 and 4. Two cases (a verrucous SCC and an advanced SCC), did not cluster with any of the other samples. We found two main biological pathways (cell communication and integrin-mediated cell adhesion) and 5 gene ontology categories (transcription regulator activity, structural molecule activity, intracellular signaling, cytoskeleton and signal transduction) of relevance to the OSCCs examined. Results from the QRT-PCR verified the results from the microarray experiment. This study provides valuable information on gene expression profile of OSCCs of habitual users of BQ from Sri Lanka. Of particular interest were the list of genes of known and unknown functions and the two biological pathways that we suggest as candidate genes in oral cancers associated with BQ chewing in Southeast Asia, in particular Sri Lanka. The suggested candidate genes might be used as molecular biomarkers in the early detection of the alarming problem of OSCCs in Southeast Asia in association with BQ use. These findings provide valuable information that might help in the selection of possible biomarkers that can be used in early detection of the alarming problem of oral cancer in Southeast Asia.

Introduction

Of the 500,000 new cases of oral squamous cell carcinomas (OSCCs) reported annually, 62% occur in developing countries with the highest rates reported in Sri Lanka and other parts of Southeast Asia (1). In this part of the globe, an estimated 600 million people are reported to chew areca nut and betel quid (BQ) (2). In Sri Lanka, OSCC is linked to the habit of BQ use (3). Areca nut (Areca catechu), the major component of BQ, has been found to contain several alkaloids that give rise to nitrosamines, some of which are carcinogenic (4). It has been suggested that BQ-specific nitrosamines (BQ-SNAs) may act as an adjunct to tobacco-specific nitrosamines (TSNAs), implicated as an etiologic factor for OSCCs (5). BQ-SNAs includes N-nitroso-guvacoline (NG) and 3-(methyl-N-nitrosamino) propionitrile (MNPN) that is also found in tobacco (6). In Sri Lanka, fresh areca nut, slaked lime from seashells, betel leaf and dried (or processed) tobacco are chewed, with the quid placed between the cheek mucosa, the lower gingival and sulcus and sometimes retained during

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sleep (2). Buccal mucosa represents the primary site for OSCC among betel quid chewers (2), contrary to tongue cancer, which represents the primary site of cancers in Western countries where cigarette smoking and heavy alcohol consumption are the main causative factors (7). Molecular mechanisms and steps of carcinogenesis in betel quid induced OSCC may differ from those caused by cigarette smoking and alcohol use (8).

Development of OSCC is a multi-step process involving genes related to cell cycle, growth control, apoptosis, DNA damage response and other cellular regulators (9). Understanding the genetic processes and biological pathways involved in the development of OSCC might lead to valuable information that might improve disease classification, early detection and diagnosis, as well as therapeutic planning and drug development (10,11). Microarrays represent a promising tool that makes it possible to explore the expression profile of thousands of genes simultaneously, at the RNA level (12,13). In the literature, there are several microarray studies on OSCCs with promising findings (12-14). Although the influence of life-style factors such as tobacco, alcohol use and nutrition are important to consider in the causation of OSCCs, there is a wide inter-individual difference(s) in susceptibility to chemical carcinogens (15). Since BQ is associated with OSCC development, we applied the 35k human oligo-microarrays (produced and supplied by the Norwegian Microarray Consortium, www.mikromatrise.no) to examine the gene expression profile in 15 cases of OSCCs from Sri Lanka with their corresponding pair-wise normal controls and correlated the findings to patient's clinicopathological parameters.

Materials and methods

Patients. Primary samples (n=15) of OSCCs and their corresponding pair-wised normal controls were acquired from consecutive patients (average age 58.8 years; range 43 to 70; SD ± 8.68) with previously untreated OSCCs operated on at the Department of Maxillofacial Surgery, University Dental School at Peradeniya, Sri Lanka. The Ethics committee at the University of Peradeniya approved the study and written consent was obtained from the participating subjects. Normal control samples were obtained either from the contralateral side of the mouth or from an area that was at least 4 to 5 cm away from the cancerous tissue and was macroscopically normal. After surgery, tissue samples (malignant and normal) were immediately submerged in the tissue storage and RNA stabilization solution, RNAlater™ (Ambion, Inc., Woodlands, TX, USA) and dispatched to the Department of Biomedicine at the University of Bergen, where they were stored at -20°C until RNA purification and microarray experiments.

All tumors were staged following the 1987 UICC staging system and had their histopathologic diagnosis confirmed by two of the authors (SW/SOI) using either fresh frozen/or 10% formalin-fixed, paraffin-embedded tissue sections stained with haematoxylin and eosin (H&E). The tumors were histologically graded into high, moderate or poorly differentiated carcinomas (16). To rule out gene expression alterations because of stromal cell contamination, we confirmed pathologically that each tumor specimen contained ≥70% cancer cells by analysis of the corresponding H&E-stained sections. For all the

patients, data on clinicopathological parameters, such as information on betel quid chewing, were available (Table I).

Tissue samples and RNA exctraction. Total RNA was extracted from both tumor and normal controls using TRIzol® reagent (Gibco BRL, Carlsbad, CA, USA)/RNeasy Fibrous Tissue kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Quality and quantity of the RNA were determined spectrophotometrically with a Beckman DU®530 Life Science Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) and by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

cDNA synthesis, hybridization and scanning. Synthesis and labeling of the cDNA was carried out using Fairplay Microarray Labelling kit (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions. Synthesized cDNA was labeled with Cy™3 (normal cDNA) and Cy™5 (tumor cDNA) monoreactive dyes (Amersham Biosciences, GE Health Care) and samples were hybridized to the human oligonucleotide microarrays containing 34,580 oligonucleotid probes (The Human Genome Oligo Set Version 3.0; Operon Biotechnologies Inc., Huntsville, AL, USA) representing human genes and gene transcripts, printed on Corning Ultra GAPS slides at the Norwegian Microarray Consortium (www.mikromatrise.no). Labeled cDNA was hybridized on the Ventana Discovery® XT System (Ventana Medical Systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Slides were scanned by Agilent DNA Microarray Scanner BA (Agilent Technologies, Palo Alto, CA, USA) and the microarray data were stored as tiff format images. The images were further analyzed with GenePix Pro v5.0 (Molecular Devices Corp., Sunnyvale, CA, USA) where bad spots and spots not found were flagged and the final results containing all statistical values were stored as a gpr-file.

Statistical analysis. Image quantization files obtained from GenePix Pro were processed and merged into a gene expression matrix using J-Express Pro software package (version 2.6; www.molmine.com) (17). Each array was first pre-processed separately by performing the following steps: i) Spots flagged by Genepix ('bad', 'absent' or 'not found', -100, -75 and -50, respectively) were filtered; ii) and in order to avoid extreme ratios in spots where only one of the channels had a significant signal, a flooring step was applied where intensity values below 30 was set to 30, thereby eliminating unwanted high ratios for spots with intensity near zero; iii) a global lowess normalization was applied to all values left after the filtering step. Thereafter, all in-array replicate spots were merged by a median statistics and inserted into a gene expression matrix where each row corresponds to a gene, each column corresponds to a patient and each cell represents the log (base 2) ratio value for the tumor versus the normal control for one gene in one patient. Since the expression matrix will contain cells with no values (missing values), genes for which more than half of the patients (arrays) had no value were removed. Missing values were set to zero, thus avoiding their contribution in indicating up- or downregulation status of a gene in tumor versus normal control. To prepare the

Table I. Clinicopathological data of the 15 patients included in the study.

Patients' clinicopathological data

Sample no	Tumor site	TNM	Stage	Clinical appearance	BQ	Smoking	Alcohol
1	Alveolus	T2 N0 M0	2	Verrucous	++	++	++
2	Buccal	T1 N0 M0	1	Exophytic	++	-	-
3	Alveolus	T2 N1 M0	3	Growth	++	-	-
4	Floor	T2 N1 M0	3	Growth	++	++	++
5	Buccal	T4 N1 M0	4	Growth	++	-	-
6	Alveolus	T4 N1 M0	4	Growth	++	-	-
7	Alveolus	T2 N1 M0	3	Growth	++	++	++
8	Buccal	T4 N1 M0	4	Exophytic/ulcer	++	++	-
9	Lateral tongue	T2 N0 M0	2	Growth	-	++	++
10	Buccal	T2 N0 M0	2	White/red ulcer	-	+	++
11	Buccal	T4 N1 M1	4	Growth	++	++	++
12	Alveolus	T4 N0 M0	4	Growth	++	-	-
13	Buccal	T2 N0 M0	2	Verrucous	++	++	+
14	Retromolar	T4 N0 M0	4	Ulcer	++	-	-
15	Buccal	T2 N0 M0	2	White/ulcer	++	++	+

TNM, tumor grading Broder's classification system; ++, regular (daily); +, occasionally; -, non-user.

expression matrix for array comparison, we applied scale normalization to reduce differences in expression spread. For finding a gene with a significant difference between tumor and normal control, the relative difference in gene expression d $(i)=M(i)/(SE(i)+s)$ was used where $M(i)$ is the mean log ratio for gene i, $SE(i)$ is the standard deviation of the gene's log ratios and s is an added constant for all genes. In words, $d(i)$ is a student's t-statistics with a fudge factor s which corrects for under-estimated variances resulting in a higher weight to high average fold change versus low variance that can be justified by the noisy nature of microarray experiments. In our case and as suggested by Efron et al (18), a0 is set to a 90 percentile of all gene standard errors $SE(i)$.

Since all tumors were labeled with Cy5 and the corresponding normal controls were labeled with Cy3, we used as a control an additional set of hybridizations of 5 pairs of experiments that included primary keratinocytes, dysplastic oral epithelial cell line, OSCC cell line (SCC-25) and two metastatic OSCC cell lines (OSC-2 and G6) that were hybridized twice with a dye swap experiment using the same arrays and identical experimental protocols. This was done to find out whether genes found as differentially expressed were due to dye swap effect or were due to the disease status. In our case, we hypothesized that a gene-specific dye effect would give genes with high s-scores in this matrix since a bias for one gene will give a higher signal with one of the dyes and will have this as a result. Therefore, we have chosen a very low threshold of 0.5 for the s-score, which has resulted in a list of 1276 genes with a possible dye effect.

For the tumor expression matrix, we selected genes with an s-score above 1.0 and obtained 461 genes as differentially expressed either between the tumors and normal controls or alternatively, as a result of the dye effect. To remove genes affected by a dye effect, we removed all genes for which array

vendor had reported a possible dye effect and also genes with an s-score above 0.5 in the dye swap expression matrix, resulting in 263 genes that we believe are differentially expressed between tumors and normal controls. We further performed a permutation experiment and generated 1,000 permuted matrices. Each permuted matrix contains the 12034 genes in the original unfiltered data set and the permutation was performed by flipping the sign of each column with a probability of 0.5. Averaged over the 1,000 permuted matrices, our analysis generated 0.47 genes per matrix with a d-score above 1.0 resulting in a false discovery rate of 0.001.

To search for changed biological systems, mapping of the selected genes was first done to a Gene Ontology (GO) Directed Acyclic Graph (DAG) and thereafter comparison of the number of the selected genes (263) in GO terms was done to the number of the genes in a GO DAG based on all genes left from low-level data preparation (12034 genes). We used the Fisher-Irwin exact test to calculate a p-value for all GO terms using a p-value cut-off at 0.05. All terms with less than three selected genes and enrichment score (ratio of the relative frequency of genes from a GO-term in the selected set to the relative frequency of genes from the same term in the full set) below 2 were removed. We searched for genes related to the same biological pathway by performing a KEGG (19-21) (Kyoto Encyclopedia of Genes and Genomes, www.KEGG. com) analysis. We also used the Fisher-Irwin exact test to find statistically significant pathways.

Hierarchical clustering, based on Pearson correlation and average-linkage (WPGMA), was performed to cluster patients with similar gene expression profiles.

Quantitative real-time RT-PCR. To validate gene expression profile for selected candidate genes, real-time quantitative RT-PCR was performed for nine genes: *GJA1*, *MMP1*,

Table II. List of the probes for the genes analyzed by QRT-PCR.

QRT-PCR probes

Gene	Assay ID	Reporter sequence
MMP1	Hs00233958_m1	TAAAGACAGATTCTACATGCGCACA
COL4A1	Hs00266237_m1	CCTCCAGGCCTCCCTGTACCTGGGC
GJA1	Hs00748445_s1	GACCAGTGGTGCGCTGAGCCCTGC
HAS3	Hs00193436_m1	TGGACTACATCCAGGTGTGCGACTC
ANGPTL4	Hs00211522_m1	CTGCACCGGCTGCCCAGGGATTGCC
CD47	Hs00179953_m1	GGCGTGCTGCGGATCAGCTCAGCTA
BPIL1	Hs00257918_m1	AGCATTGAGCTACGTGTCTGAAATT
WFDC2	Hs00196109_m1	CACCCTAGTCTCAGGCACAGGAGCA
S100A1	Hs00196704_m1	CCAGGCCAACCGTGCACTGCTGCAA
ACTB	Hs99999903_m1	GCCTCGCCTTTGCCGATCCGCCGCC

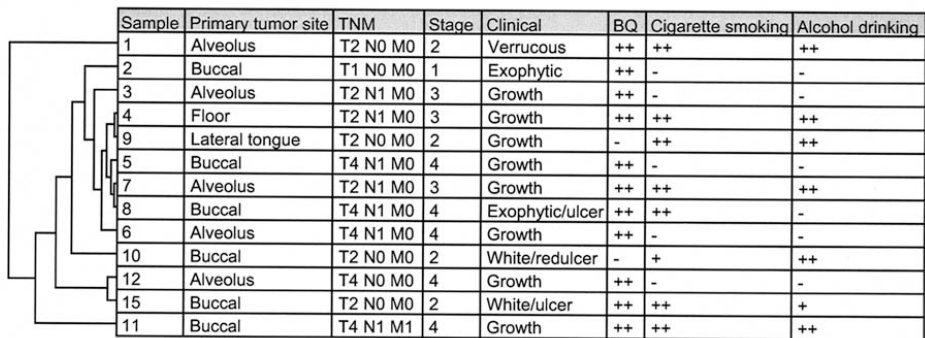| Sample	Primary tumor site	TNM	Stage	Clinical	BQ	Cigarette smoking	Alcohol drinking
1	Alveolus	T2 N0 M0	2	Verrucous	++	++	++
2	Buccal	T1 N0 M0	1	Exophytic	++	-	-
3	Alveolus	T2 N1 M0	3	Growth	++	-	-
4	Floor	T2 N1 M0	3	Growth	++	++	++
9	Lateral tongue	T2 N0 M0	2	Growth	-	++	++
5	Buccal	T4 N1 M0	4	Growth	++	-	-
7	Alveolus	T2 N1 M0	3	Growth	++	++	++
8	Buccal	T4 N1 M0	4	Exophytic/ulcer	++	++	-
6	Alveolus	T4 N1 M0	4	Growth	++	-	-
10	Buccal	T2 N0 M0	2	White/redulcer	-	+	++
12	Alveolus	T4 N0 M0	4	Growth	++	-	-
15	Buccal	T2 N0 M0	2	White/ulcer	++	++	+
11	Buccal	T4 N1 M1	4	Growth	++	++	++

Figure 1. Hierarchical clustering of the 263 genes found as differentially expressed between tumors and normal controls and their possible correlation to the patients clinicopathological data.

COL4A1, *HAS3*, *CD47* and *ANGPTL4* (all were upregulated), *S100A1*, *BPIL1* and *WFDC2* (all were downregulated). These genes were selected for verification because four of them (*GJA1*, *MMP1*, *COL4A1* and *S100A1*) were previously reported in oral cancer, while the rest (*HAS3*, *CD47*, *ANGPTL4*, *BPIL1* and *WFDC2*) were not. Aliquots of the same RNA (200-300 ng) used for the microarray hybridization was also used for synthesis of the cDNA, performed with High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA), following the manufacturer's instructions. Real-time PCR was performed with probes for each gene (listed in Table II) using the ABI 7900 HT (Applied Biosystems) and 384 well optical plates (ABI). Each reaction contained 1 μl cDNA, 5 μl 2x TaqMan Universal Master mix (Applied Biosystems), 0.5 μl Taqman AOD probe and H_2O to a final volume of 10 μl and was run in triplicate. Cycling parameters were 95ºC for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Serial diluted standards were run on the same plate and the relative standard curve method was used to calculate the gene expression as described elsewhere. ß-actin was used as an endogenous normalization control to adjust for unequal amounts of RNA.

Results

Among the 15 patients studied [3 (20%) females and 12 (80%) were males], 13 were regular betel quid chewers. Six (40%) were both cigarette smokers and alcohol users, while one (7%) patient was a regular smoker without other habits. The two (14%) non-betel quid chewers smoked and drank alcohol regularly. Two of the 15 cancers were verrucous carcinomas while all the others were of squamous cell type.

Gene expression profile. Determination of the gene expression profile of the 15 Sri Lankan patients, by hybridizing the cDNA from the pair-wise normal and tumor samples to the 35k human oligo microarray slides, resulted in a set of 263 genes found as differently expressed between tumors and normal controls. Of these, 190 (72%) genes (Table IIIA) were found as upregulated and 73 (28%) (Table IIIB) were found as downregulated.

Since there are many genes reported as differentially expressed in OSCCs, we searched the Cancer Genome Anatomy Project (CGAP, http://cgap.nci.nih.gov/) for genes related to head and neck cancer, by using the Gene Library

Table IIIA. Genes upregulated in tumor versus normal controls.

Gene name/function	Gene symbol	Oligo_ID	Average fold change
Adhesion associated genes			
Cytokeratin 6A	KRT6A	H300014335	19.452
Keratin 17	KRT17	H200000678	14.011
Collagen I, alpha 1	COL1A1	H300015580	8.804
Periostin, osteoblast specific factor	POSTN	H300000923	8.632
Collagen IV, alpha 1	COL4A1	H200012184	7.971
Chloride channel	CLCA2	H200015766	6.891
Odd Oz/ten-m homolog 2	ODZ2	H200008152	6.394
Hyaluronan synthase 3	HAS3	H200007172	6.024
Keratin 5	KRT5	H300019235	5.745
Myosin IB	MYO1B	H200012339	5.382
Ladinin 1	LAD1	H200002708	4.909
Serine proteinase inhibitor	SERPINH1	H200001857	4.709
Thy-1 cell surface antigen	THY1	H200012556	4.017
Molecule interacting with Rab13	MIRAB13	H300008711	3.977
Bicaudal D homolog 2 (Drosophila)	BICD2	H200019300	3.578
CD47 antigen	CD47	H200006967	3.317
Epithelial protein lost in neoplasm beta	EPLIN	H200001929	3.263
Development and differentiation enhancing factor 1	DDEF1	H200001924	3.076
Osteonectin	SPARC	H200011770	3.027
Actinin, alpha 1	ACTN1	H200012175	2.915
Dipeptidyl-peptidase 3	DPP3	H300022630	2.702
Hypothetical protein similar to actin	ARPC5L	H200018870	2.561
CD 44 antigen	CD44	H300019814	2.542
Eukaryotic translation initiation factor 4A	EIF4A1	H200012822	2.399
Plectin 1	PLEC1	H200006713	2.302
Integrin, beta 1	ITGB1	H300017353	2.207
Actin, gamma 1	ACTG1	H300006234	2.138
Tropomyosin 4	TPM4	H300013721	2.088
Angiogenesis			
Matrix metalloproteinase 1	MMP1	H200007011	20.586
Matrix metalloproteinase 3	MMP3	H200007019	7.764
Plasminogen activator, urokinase	PLAU	H200006377	7.280
Endothelial cell growth factor 1	ECGF1	H200005893	7.054
Hexabrachion	TNC	H200009494	6.792
Interleukin 8	IL8	H200000156	5.645
Angiopoietin-like 4	ANGPTL4	H300021145	4.930
Jagged 1	JAG1	H300002958	4.773
Tryptophanyl-tRNA synthetase	WARS	H300012785	4.845
Hypoxia-inducible factor 1, alpha subunit	HIF1A	H200014761	2.620
Apoptosis			
Caspase 1	CASP1	H200000628	7.589
Cofilin 1 (non-muscle)	CFL1	H200008422	2.388
Cell-cell signaling			
Interferon, alpha-inducible protein (clone IFI-15K)	G1P2	H300006942	18.761
Interferon, alpha-inducible protein 27	IFI27	H200017325	8.509
Connexin 43	GJA1	H200005947	3.940
Cerebral cavernous malformation 2	CCM2	H200001861	2.626
Protein arginine methyltransferase 1	PRMT1	H300019121	2.458
Cell growth regulation			
Solute carrier family 16	SLC16A1	H200006037	7.932
Bone marrow stromal cell antigen 2	BST2	H200012083	7.175
Serpin peptidase inhibitor	SERPINB5	H300020178	6.169

Table IIIA. Continued.

Gene name/function	Gene symbol	Oligo_ID	Average fold change
Caveolin 2	CAV2	H200013312	4.587
N-myc (and STAT) interactor	NMI	H300001976	4.525
Guanylate binding protein 2	GBP2	H200007999	4.412
Retinol binding protein 1	RBP1	H300001638	4.261
Basonuclin 1	BNC1	H300005994	4.252
Breast cancer metastasis suppressor 1	BRMS1	H300002128	3.891
RAB31, member RAS oncogene family	RAB31	H200015253	3.864
EBNA1 binding protein 2	EBNA1BP2	H300005177	3.554
Caveolin 1	CAV1	H200005908	3.459
Polo-like kinase 2	PLK2	H300012787	3.348
Karyopherin alpha 2	KPNA2	H300007884	3.317
Stratifin	SFN	H300006188	3.275
Ribosomal protein S6 kinase	RPS6KB2	H300007163	2.963
Non-metastatic cells 1	NME1	H200012135	2.963
Proliferation-associated 2G4	PA2G4	H300010406	2.875
Proteasome (prosome, macropain) 26S subunit	PSMD1	H200000856	2.782
Exostoses (multiple) 2	EXT2	H200006075	2.593
Suppressor of G2 allele of SKP1	SUGT1	H200001007	2.354
RNA binding motif	RBMS1	H300004002	2.255
Nitric oxide synthase interacting protein	NOSIP	H300022154	2.188
Immune response			
Ig gamma-4 chain C region	IGHG4	H300000934	10.716
Guanylate binding protein 1	GBP1	H200005495	9.735
2'-5'-oligoadenylate synthetase 2	OAS2	H300002591	8.434
Transporter 1, ATP-binding cassette	TAP1	H300021278	5.455
Cathepsin C	CTSC	H300018822	4.684
Interferon, alpha-inducible protein	G1P3	H200016555	4.436
Proteasome activator subunit 2	PSME2	H300007599	3.071
Apolipoprotein L, 3	APOL3	H200015760	3.048
Interferon induced transmembrane protein 2	IFITM2	H300004907	2.941
Tumor necrosis factor, alpha-induced protein 1	TNFAIP1	H200006250	2.933
Integral to membrane			
Leucine-rich repeat-containing protein 8A	LRRC8	H200008145	3.525
Intracellular signaling			
Rag C protein	RRAGC	H200011726	3.678
Ras homolog gene family, member D	RHOD	H200002449	3.101
Diacylglycerol kinase	DGKZ	H200017225	2.975
Cornichon homolog 4	CNIH4	H300011960	2.943
Intracellular transport			
Tubulin beta-5	TUBB6	H200017078	3.826
Nuclear transport factor 2	NUTF2	H300010247	2.337
Metabolism			
Cathepsin L2	CTSL2	H200010239	5.169
Similar to glucosamine-6-sulfatases	SULF2	H200004653	5.082
GM2 ganglioside activator protein	GM2A	H200009479	4.964
Selenoprotein X, 1	SEPX1	H200017458	3.870
Carbohydrate (N-acetylglucosamine-6-O)	CHST2	H200001687	3.864

Table IIIA. Continued.

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Gene name/function	Gene symbol	Oligo_ID	Average fold change
Heat shock 105 kDa/110 kDa protein 1	HSPH1	H300019008	3.845
Prion protein (p27-30)	PRNP	H200005977	3.688
Thioredoxin-like 5	TXNL5	H200005936	3.497
Homo sapiens cDNA FLJ30135 fis, clone BRACE2000061	BLOC1S2	H200004282	3.230
Small nuclear ribonucleoprotein polypeptide G	SNRPG	H300006350	3.104
Adenosine kinase	ADK	H200010643	3.042
Mitochondrial ribosomal protein L15	MRPL15	H200002720	2.947
Heat shock 70 kDa protein 4	HSPA4	H300022500	2.895
Mitochondrial ribosomal protein L17	MRPL17	H200001870	2.811
Small nuclear ribonucleoprotein polypeptide A'	SNRPA1	H300000296	2.670
Similar to peptidylprolyl isomerase (cyclophilin)-like 1	PPIL1	H300000902	2.664
Prefoldin subunit 2	PFDN2	H200009859	2.650
Small nuclear ribonucleoprotein polypeptide B"	SNRPB2	H200006957	2.626
Chaperonin containing TCP1, subunit 5 (epsilon)	CCT5	H200000420	2.578
Mitochondrial ribosomal protein L14	MRPL14	H300010300	2.545
Similar to peptidylprolyl isomerase (cyclophilin)-like 1	PPIL1	H300002507	2.499
Peptidylprolyl isomerase A (cyclophilin A)	PPIA	H300005405	2.382
LSM1 homolog, U6 small nuclear RNA associated	LSM1	H200011772	2.289
Mitochondrial ribosomal protein S12	MRPS12	H200001864	2.273
Nucleolar protein family A, member 3	NOLA3	H300001534	1.955
Proteasome (prosome, macropain) 26S subunit	PSMC1	H200000913	1.858

Oncogene

V-Ha-ras Harvey rat sarcoma viral oncogene homolog	HRAS	H300018518	3.298
Met proto-oncogene (hepatocyte growth factor receptor)	MET	H300013056	3.141
V-jun sarcoma virus 17 oncogene homolog (avian)	JUN	H200006516	1.926

Signal transduction

Signal transducer and activator of transcription 2, 113 kDa	STAT2	H300010660	4.436
Lymphocyte antigen 6 complex, locus E	LY6E	H200006433	3.491
Tyrosine kinase, non-receptor, 2	TNK2	H300022337	2.292

Transcriptional regulation

Superoxide dismutase 2, mitochondrial	SOD2	H200018923	4.335
Interferon regulatory factor 7	IRF7	H300021511	3.581
BolA-like protein 2	BOLA2	H300001390	3.158
SUB1 homolog (S, cerevisiae)	SUB1	H300000824	3.127
BTB and CNC homology 1, basic leucine zipper Transcription factor 1	BACH1	H200013953	2.967

Ubiquitin cycle

Ubiquitin-conjugating enzyme E2S	UBE2S	H300007235	4.858
Proteasome (prosome, macropain) subunit, alpha type, 1	PSMA1	H200006899	3.307
Proteasome (prosome, macropain) subunit, alpha type, 4	PSMA4	H200016300	3.206
Proteasome (prosome, macropain) subunit, beta type, 2	PSMB2	H300007846	3.037
Ring finger protein 149	RNF149	H300001827	2.706
Ubiquity-conjugating enzyme E2L 3	UBE2L3	H300004594	2.410
Ring-box 1	RBX1	H200017552	2.236

Genes with other functions

SRY (sex determining region Y)-box 20	SOX15	H200010702	6.417
BA13B9,3 (Novel protein similar to KRT8)	KRT8L1	H300008020	4.819
TLH29 protein precursor	FAM14A	H200010658	4.498
Hematological and neurological expressed 1	HN1	H300005778	4.229
Nuclear RNA helicase, DECD variant of DEAD box family	DDX39	H200018834	3.966
HSPC160 protein	ORMDL2	H200002233	2.948

Table IIIA. Continued.

Gene name/function	Gene symbol	Oligo_ID	Average fold change
CDW92 antigen	CDW92	H200008388	3.832
Brix domain containing 2	BXDC2	H300020946	2.822
Solute carrier family 7, member 5	SLC7A5	H200014300	2.783
Signal peptidase complex subunit 3 homolog	SPCS3	H200004559	2.667
Protein phosphatase 1, regulatory (inhibitor) subunit 14B	PPP1R14B	H300013966	2.471
Coiled-coil-helix-coiled-coil-helix domain containing 1	CHCHD1	H300005351	2.329
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21	DDX21	H200007834	1.996
Genes with unknown function			
Epithelial stromal interaction 1 (breast)	EPSTI1	H200019973	7.766
Family with sequence similarity 83, member A	FAM83A	H200016387	6.537
Hypothetical protein MGC4677	LOC541471	H300002112	4.692
Melanoma-derived leucine zipper, extra-nuclear factor	MLZE	H200013079	4.471
Hepatitis delta antigen-interacting protein A	CCDC85B	H200005619	3.048
Family with sequence similarity 49, member B	FAM49B	H300020526	2.927
Chromosome 19 open reading frame 22	C19orf22	H200001829	2.891
COBW domain containing 1	CBWD1	H300020976	2.690
Hypothetical protein FLJ10350	RPRC1	H200008278	2.685
KIAA0842 protein	KIAA0842	H200005962	2.680
Hypothetical protein MGC22793	MGC22793	H300005475	2.312
TPA regulated locus	TPARL	H200015568	2.186
Unknown	-	H300016521	16.121
Unknown	-	H300000399	11.819
Unknown	-	H300016456	10.856
Unknown	-	H300022261	9.555
Unknown	-	H300009062	6.931
Hypothetical gene supported by AK023162	-	H200001742	6.817
Unknown	-	H300014044	6.517
Unknown	-	H300008061	5.898
Unknown	-	H300006085	5.625
Unknown	-	H300000084	5.119
Q5T7K4_HUMAN	-	H300002726	4.300
Hypothetical protein MGC4677	-	H300002113	4.006
Unknown	-	H200001872	3.982
FLJ46072	-	H300002815	3.688
Unknown	-	H300000271	3.266
Unknown	-	H300006679	2.888
Unknown	-	H300021607	2.751
Unknown	-	H300003367	2.620
Unknown	-	H300008569	2.549
Hypothetical protein DKFZp586I1420	-	H200011821	2.523
Unknown	-	H300001105	2.479
Unknown	-	H300000638	2.439
Unknown	-	H300012353	2.439
Unknown	-	H300000861	2.325
Unknown	-	H300009853	2.256
Unknown	-	H300001828	2.242
Unknown	-	H300022664	2.216
Unknown	-	H300003465	2.196
13kDa differentiation-associated protein	-	H200004675	2.162
Unknown	-	H300009555	2.158
Unknown	-	H300009420	2.045
Unknown	-	H300008478	1.987
Unknown	-	H200003368	1.971
Unknown	-	H300003763	1.867

Summarizer tool and the following search criteria: Organism: Homo sapiens; Library Group: CGAP Libraries; Tissue type: Head and neck; Library Preparation: Any; Tissue Histology: Cancer; Library Protocol: Any. We compared the differentially expressed genes found in our study with the search results (2,500 genes involved in head and neck cancer) and found that 66 of the genes [such as *COL4A1*, *COL1A1*, *PLAU*, *MMP1*, *MMP3*, *ITGB1*, *SPARC* (upregulated), *SLPI*, *EEF1A1* and *KRT19* (downregulated), among others] matched results from the CGAP search. These genes have also been reported in previous studies related to gene expression profiles in oral cancer (14). The genes that were neither listed in the CGAP nor previously reported in OSCCs included *CD47*, *UBE2L3*, *RPS6KB2*, *APOL3*, *RRAGC* (upregulated), *AZGP1*, *CLN3* and *XBP1* (downregulated). To obtain information on gene function, we searched the CGAP database using the Gene Finder tool and the Gene Ontology Browser. Genes of known function (192 of the 263 genes) were found to be related to cellular processes like cell adhesion, cell signaling, angiogenesis, metastasis and metabolism.

To determine the significant changes related to the biological systems, a GO DAG analysis was performed, which resulted in the following terms: transcription regulator activity, structural molecule activity, intracellular signaling cascade, cytoskeleton and signal transduction.

Hierarchical clustering. Hierarchical clustering of the 15 samples using the 263 genes found, resulted in several subgroups (Fig. 1). Sample number 1 and 11 (verrucous SCC and an advanced SCC), did not cluster with any of the other cases. The remaining cases clustered in several subgroups, such as samples with tumors of different TNM status, BQ chewers and non-BQ chewers, smokers, non-smokers, drinkers and non-drinkers. Interestingly however, tumors of stage three and four showed a tendency to group together, although one of the samples (number nine, a tumor of stage 2) clustered with these tumors, which might indicate a tendency towards developing an aggressive behavior though presented as stage 2. Although this is an interesting observation, analysis of additional cases is necessary. All patients were smokers and/or BQ chewers with varying types of alcohol habits. The two non-BQ chewers grouped differently, although their tumors were of stage 2. One of the samples (non-betel quid user) clustered in a large subgroup, while the other separated from the rest in a subgroup on a higher level. Patient number 2, diagnosed with a stage 1 tumor, was also distinguishable from the other patients and grouped separately.

KEGG pathway analysis. The KEGG pathway analysis (performed with the 192 genes with known functions), showed six pathways where a significant number of the genes found were included. The predominant pathways found were cell communication and integrin-mediated cell adhesion (Fig. 2) where 10 and 11 genes were represented, respectively.

Quantitative real-time RT-PCR. Gene expression profile was validated by quantitative reverse transcription for nine genes. We used ß-actin for normalization and determination of the results. A good correlation was found between the microarray data and the RT-PCR results (Fig. 3).

Discussion

In this study, we analysed gene expression profile in 15 cases of OSCCs from Sri Lanka and correlated the findings to the clinicopathological parameters. We identified 263 genes as differentially expressed between tumors and normal controls where 190 (72%) were upregulated and 73 (28%) were downregulated. Of the genes found, 192 (73%) were of known functions, where 66 of these genes (34%) (such as *COL1A1*, *COL4A1*, *MMP1*, *TNC* and *PLAU*) were previously reported in the CGAP database (http://cgap.nci.nih.gov/). The 192 known genes were found to be implicated in cellular processes like cell growth, cell proliferation, cell signaling and angiogenesis that were suggested to play an important role in oral tumorigenesis (9,22,23). We selected nine genes (*COL4A1*, *GJA1*, *MMP1*, *HAS3*, *CD47*, *ANGPTL4*, *S100A1*, *BPIL1* and *WFDC2*) and verified their expression with quantitative real-time PCR. Both up- and downregulated genes were selected, as well as genes that were either previously reported or not reported in OSCCs.

COL4A1, found as upregulated in this study, is the main constituent of the extracellular matrix (ECM) and the basement membrane (BM) (24). It has been suggested that increased collagen synthesis is related to the use of betel quid and oral submucous fibrosis - a precursor condition to oral cancer (25). Tsai *et al* (26) studied gene expression profile in oral cancers from betel quid chewers in Taiwan and reported an increase in *COL4A1* expression. Other studies in OSCCs have also reported an increased expression of this gene (14). In Taiwan, BQ is used without tobacco, but in Sri Lanka tobacco is commonly added to BQ (2). Our findings of increased *COL4A1* expression in the biopsies examined from Sri Lanka are in agreement with the findings from Taiwan, suggesting a possible role of BQ in the upregulation of *COL4A1*. These results indicate that *COL4A1* might be considered as a possible biomarker in BQ related lesions, but further confirmatory studies are needed.

HAS3, showing increased expression in this study, encodes the enzyme hyaluronan synthase 3, one of the three enzymes involved in synthesis of hyaluronan, an ECM related glyco-saminoglycan associated with proliferation and migration (27). Hyaluronan has been found to interact with *CD44* (found as upregulated in our study) and was suggested to be implicated in tumor growth and metastasis. There are no reports of *HAS3* in relation to oral cancer and this is also the case with *CD47*, a gene encoding a cell adhesion protein (28).

We detected an upregulation of *ANGPTL4* (angiopoietin-like 4, previously not reported in oral cancer), a gene induced by hypoxia and grouped under angiogenesis-related genes (pro-angiogenic) (29). *MMP1* is a metalloproteinase involved in the breakdown of the ECM during angiogenesis, invasion and metastasis (30,31). Our study showed upregulation of *MMP1*, which is in agreement with other experiments in oral cancer (14,26,32). *GJA1* (Connexin43, a gap junction constituent involved in intercellular communication) (33), has been suggested to play an important role in the regulation of cell growth and is associated with cancer development (33). Our findings of upregulation of this gene are supported by one other previous study on OSCC (34). *S100A1*, found as downregulated in our study, is a member of the *S100* gene

Table IIIB. Genes downregulated in tumor versus normal controls.

Gene name/function	Gene symbol	Oligo_ID	Average fold change
Apoptosis			
Phospholipid scramblase 3	PLSCR3	H300022882	-3.103
Ceroid-lipofuscinosis, neuronal 3, juvenile	CLN3	H300012300	-2.188
Adhesion associated genes			
Statherin	STATH	H200004368	-101.425
Alpha-2-glycoprotein 1, zinc	AZGP1	H200000021	-41.885
Keratin 19	KRT19	H200008573	-15.716
Cysteine-rich secretory protein 3	CRISP3	H200005086	-12.501
Mucin 5, subtype B, tracheobronchial	MUC5B	H300022927	-9.895
Keratin 7	KRT7	H200003337	-4.630
Cell growth regulation			
Putative cytokine high in normal-1	HIN-1	H200005487	-17.842
Tetraspan 1	TSPAN1	H200004450	-9.837
N-myc downstream-regulated gene 2	NDRG2	H200015807	-4.738
Immune response			
Secretory leukocyte protease inhibitor	SLPI	H200016308	-9.310
Immunoglobulin J polypeptide	IGJ	H300003753	-4.258
X-box binding protein 1	XBP1	H200013682	-3.134
Intracellular signaling			
S100 calcium binding protein A1	S100A1	H200009536	-3.485
Metabolism			
Phosphoglycerate dehydrogenase	PHGDH	H300006924	-5.323
Carbonyl reductase	DCXR	H200001845	-2.262
Eukaryotic elongation factor 1 alpha 1	EEF1A1	H300022640	-2.075
Eukaryotic translation elongation factor 1 gamma	EEF1G	H300009473	-1.997
Ribosomal protein L22	RPL22	H300009237	-1.929
Similar to ribosomal protein L13a	LOC387841	H300002118	-1.672
Ribosomal protein L13	RPL13	H300021124	-1.565
Proteolysis			
WAP four-disulfide core domain 2	WFDC2	H300022511	-27.078
Kallikrein 11	KLK11	H200005275	-4.398
ATG4 autophagy related 4 homolog B	APG4B	H300003574	-2.167
Signal transduction			
Calcitonin-related polypeptide, beta	CALCB	H200017136	-15.155
Protein FAM3D precursor	FAM3D	H200005421	-10.170
Tetraspanin 13	TSPAN13	H300011863	-5.236
GNAS complex locus	GNAS	H300022096	-2.367
Transcriptional regulation			
Zinc finger protein 253	ZNF253	H300008475	-2.138
Genes with other functions			
Hypothetical protein dJ726C3.2	BPIL1	H200016425	-196.506
Proline rich 4 (lacrimal)	PRR4	H300001196	-41.384
Mucin 7, salivary	MUC7	H200011272	-37.298
Trefoil factor 3 (intestinal)	TFF3	H300004783	-20.952
Transcobalamin	TCN1	H200000515	-9.862
Olfactory receptor 5T2	OR5T2	H300003770	-8.712
Hemoglobin, alpha 1	HBA1	H200016940	-8.655
Crumbs homolog 3 (Drosophila)	CRB3	H200013695	-8.483
Hemoglobin, beta	HBB	H300022339	-5.687
Defensin, beta 1	DEFB1	H200004191	-4.573

Table IIIB. Continued.

Gene name/function	Gene symbol	Oligo_ID	Average fold change
Hypothetical protein FLJ40504	FLJ40504	H300004165	-3.751
Olfactory receptor 8G5	OR8G5	H300006949	-3.561
H2A histone family, member L	HIST1H2AC	H200003862	-3.446
Lysozyme (renal amyloidosis)	LYZ	H200015514	-3.077
Transmembrane emp24 protein transport domain containing 3	TMED3	H200008352	-2.719
Reticulon 3	RTN3	H300008519	-2.583
Chromosome 1 open reading frame 8	C1orf8	H200002003	-2.359
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	ATP5G2	H200010315	-2.216
Genes with unknown function			
Hypothetical protein LOC124220	LOC124220	H200011403	-180.985
Anterior gradient 2 homolog (Xenepus laevis)	AGR2	H200010467	-37.251
Dpy-like 2 protein	DPY19L2	H300011031	-27.669
Chromosome 4 open reading frame 7	C4orf7	H300008107	-10.921
La ribonucleoprotein domain family, member 2	LARP2	H300022914	-4.066
KIAA1693	NBPF1	H300006550	-3.177
Glioma tumor suppressor candidate region gene 2	GLTSCR2	H300001487	-2.023
Unknown	-	H300007292	-98.978
Unknown	-	H300015187	-24.882
MGC34829 protein	-	H300004686	-23.621
Hypothetical protein DKFZp586L111	-	H200019198	-22.175
Unknown	-	H300011211	-18.810
Hypothetical protein LOC90113	-	H200019881	-13.858
PRO1848	-	H200007208	-6.556
Hypothetical protein DKFZp781I24108	-	H300001302	-4.690
Unknown	-	H300008514	-4.604
Unknown	-	H300005667	-4.363
Unknown	-	H300008538	-4.245
Unknown	-	H300010322	-3.754
Unknown	-	H300017074	-2.986
Homo sapiens, clone IMAGE:3633225, mRNA	-	H200020471	-1.892
Unknown	-	H300009486	-1.839
Unknown	-	H300004908	-1.783
Unknown	-	H300002725	-1.714
Unknown	-	H300000309	-1.644

family, coding for Ca^{2+}-binding proteins, suggested to play an important role in cancer progression (35). Downregulation of *S100A1* has been reported in another study on head and neck cancer (36). *WFDC2*, Wap four-disulfide core domain 2 (found as downregulated in this study), is a gene encoding a protease inhibitor and has been suggested as a tumor biomarker in ovarian carcinoma (37).

OSCC is a major health problem related to betel quid use and smokeless tobacco habits in developing countries, albeit there are few studies focusing on gene expression profile of oral cancers from these countries. There are several studies performed in OSCCs from western countries (14,32,38-40), and some of the genes found in our study to be differentially expressed correlate with findings from western countries. These include in particular *COL4A1*, *COL1A1*, *MMP1*, *PLAU*, *SPARC*, *TNC* and *KRT19*, with *MMP1* and *PLAU* as the most frequent genes. MMPs have been described as possible

biomarkers of invasion and metastasis in oral cancers (30), which might also apply for the cases examined herein. *PLAU* has been suggested to be implicated in enhanced cell proliferation and migration (41) and as a prognostic marker for relapse-free survival of OSCCs, together with its receptor *u*PAR (42). *SPARC*, or osteonectin, is also implicated in ECM breakdown (43) and has been reported to be associated with tumor progression and metastasis (44). This protein was previously reported in two studies from Sudan, where the use of oral snuff (*toombak*) is common (45,46). The frequent reports of the ECM related genes in association with OSCCs might suggest that ECM breakdown is an important process in oral carcinogenesis. This is supported by our finding of cell adhesion as one of two predominant biological pathways. *MMP1* is related to *COL4A1*, which is an important ECM constituent, suggesting that an increase in *COL4A1* expression might be related to betel quid usage as suggested by others
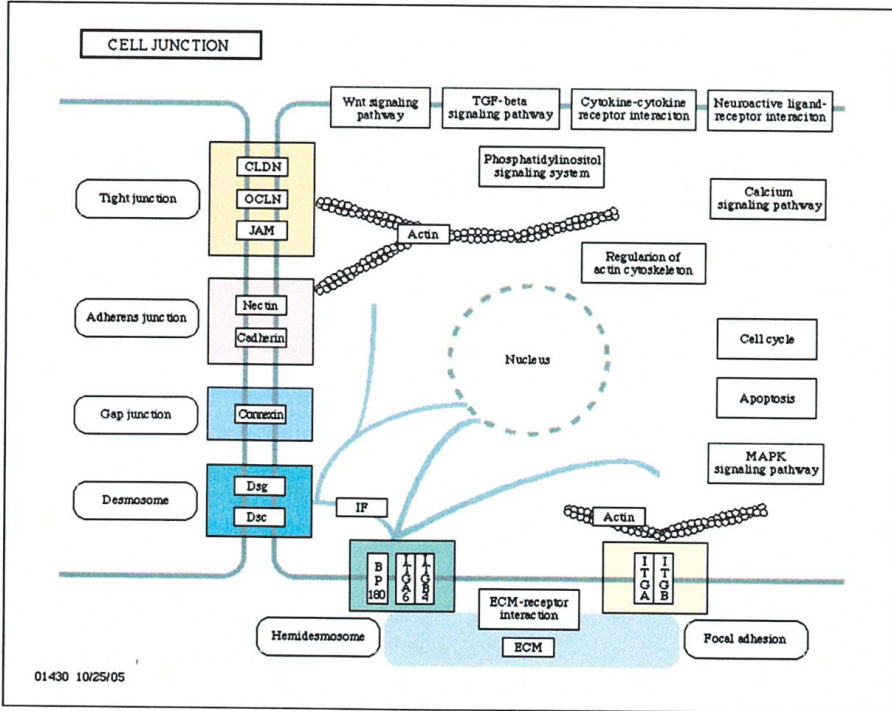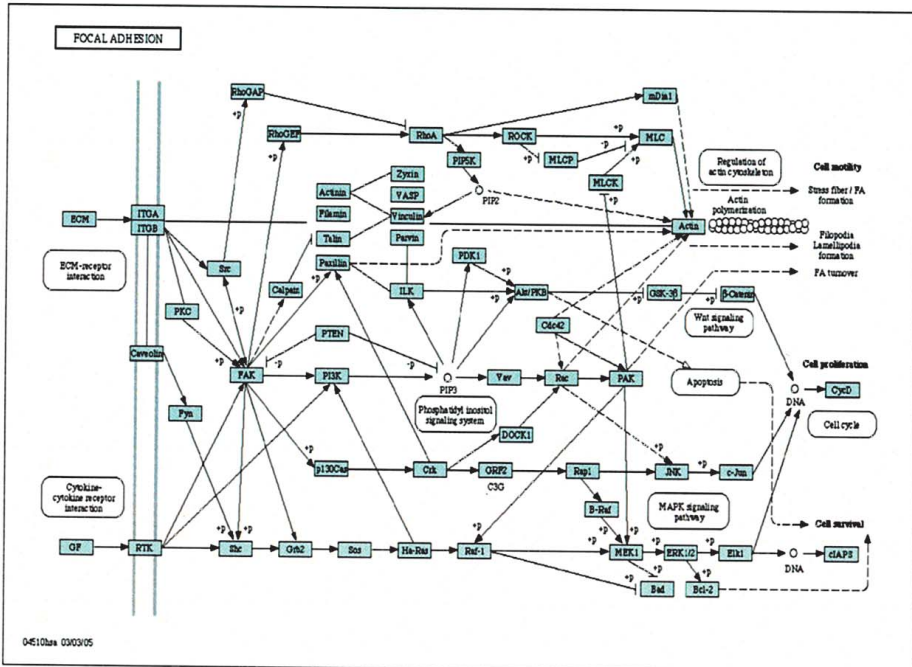
Figure 2. KEGG analysis illustrating cell communication (2A) and focal adhesion molecules (2B) pathways.
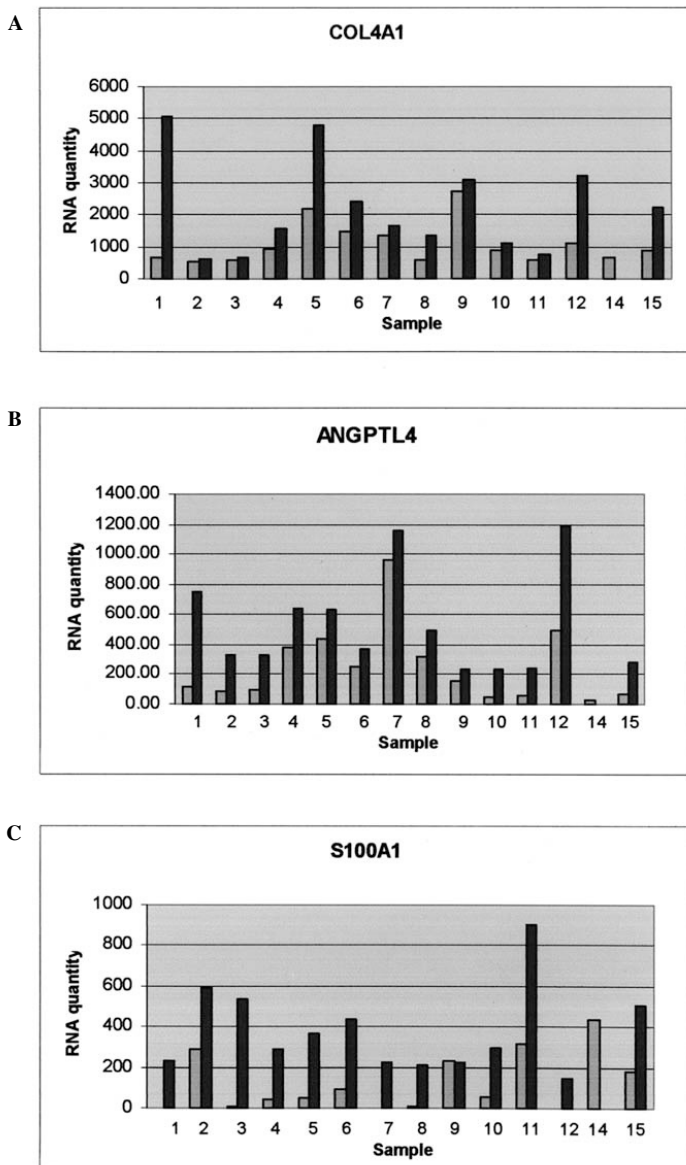
Figure 3. Association between the microarray hybridization and the QRT-PCR data. Results are presented here for COL4A1 (A), ANGPTL4 (B) and S100A1 (C). The light columns, RT-PCR data; the dark columns, microarray data.

(25,47,48). Since *PLAU* may also be related to *COL4A1* through ECM breakdown, *MMP1*, *COL4A1*, *PLAU* and *SPARC* might be suggested as possible biomarkers for OSCCs associated with betel quid chewing as a causative factor. The fact that these four genes have been consistently reported in relation to cancers of the oral cavity in Asian populations might indicate a possible relationship between their increased expression and betel quid usage.

Gene expression profiling not only offers the possibility of finding specific genes differentially expressed between cancer and normal tissue, but individual samples or subgroups can also be distinguished by determining the general profile of each sample. In our study, hierarchical clustering of the analyzed samples showed several subgroups, with the exception of one case diagnosed with a verrucous SCC - a tumor related to smokeless tobacco that is usually less

aggressive compared to other OSCCs (39,40,49). Another sample also differed from the other cases by grouping separately, and this was the only stage 1 tumor among the 15 cancers analyzed. We observed a large subgroup of samples with tumors predominately at stage 3 and 4, which might suggest that advanced tumors have a common gene expression profile compared with tumors at stage 1 and 2. Interestingly however, one other stage 2 tumor clustered with tumors of stage 3 and 4, suggesting a gene expression profile in this sample that is similar to advanced tumors. Relevance of these observations needs to be verified in larger samples. Other OSCC studies using gene expression profiles have elucidated a way of distinguishing metastasizing tumors from non-metastasizing tumors (14). Data of this kind may be valuable in predicting the outcome and risk of metastasis of different tumors, in addition to the traditional tumor classification criteria. Identification of aggressive tumors also offers the possibility of describing individual therapeutic approaches and improved treatment planning.

Further studies will therefore be necessary to confirm our findings, which might lead to a better understanding of the molecular basis of cancer development and tumor progression, particularly in relation to BQ induced oral cancers.

Acknowledgements

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