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Alterations in Gene Expression Associated with Head and Neck Squamous Cell Carcinoma Development

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Abstract. *Background: Normal keratinocytes (KC) and neoplastic cells derived from a head and neck lesion (SCC-25) were grown as organotypic raft cultures to mimic in vivo architecture in the absence of contaminating cell types. Alterations in gene expression between normal keratinocytes and a head and neck squamous cell carcinoma (HNSSC) cell line (SCC-25) were analysed using gene arrays. Materials and Methods: RNA from the organotypic raft cultures were used to probe four gene arrays. Gene expression alterations between the normal and neoplastic cells were identified and analysed using both fold differences and 2-tailed t-test. Four genes from different functional groups were used for immunohistochemical staining of patient tumours to confirm the gene array data. Results: Statistical analysis of the array data revealed 124 significantly altered genes between normal and neoplastic HNSCC cells. These gene expression alterations are associated with a variety of different functional groups and indicate the complexity of gene de-regulation associated with HNSCC. Conclusion: This study identified many novel gene alterations associated with HNSCC. The significantly altered gene alterations belong in a variety functional groups including: growth control, apoptosis and detoxication and present new targets for investigating the molecular basis of HNSCC formation.*

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer in the world (Refs. 1,2 and authors therein) and represent 5% of all malignant neoplasms worldwide (3). Although recent advances in medical science has increased 5-year survival rates for many HNSCCs, the rate of recurrence is still approximately 3% per year and mortality rates are still unacceptably high. The high rates of mortality from HNSCC coupled with the morbidity associated with HNSCC treatment highlights the need for new therapeutic strategies to treat patients with HNSCC.

In order to establish new strategies for HNSCC treatment, it is essential to have a comprehensive understanding of the tumour and the tissue environment in which it grows (both at a biological and molecular level). For instance, there is considerable evidence to indicate that the formation of head and neck squamous cell carcinomas are strongly correlated with excessive smoking and alcohol consumption (4-6). However, the precise molecular targets for these oncogenic insults are currently unclear. Whilst the identification of these molecular targets is unknown, we are starting to gain an understanding of biological dysfunctions associated with HNSCC. For example, it is accepted that HNSCC have disruptions in pathways regulating differentiation and apoptosis (7-9). However, apart from a few known gene alterations (*e.g.* cyclin D1 and epidermal growth factor receptor [EGFR]) associated with HNSCC(10-18), little is known about the molecular/genetic alterations that contribute to the altered biology of keratinocytes that results in HNSCC formation. Since neoplastic cell biology differs from that of normal cells, it is reasonable to predict that this is also reflected at the molecular level. Indeed, gross genetic alterations in HNSCC cells have been reported in many studies, some of which are indicated in Table I.

The identification of alterations in gene expression that accompany HNSCC development has been facilitated by the development of gene array and microarray technology (19-21). Earlier studies which used gene array profiling of HNSCCs have produced a large number of candidate genes

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Key Words: Keratinocyte, squamous cell carcinoma, gene array, carcinogenesis, gene expression.

Table I. *Examples of genes that are altered in head and neck squamous cell carcinomas.*

Gene	Alterations	References
EGFR	overexpression	10,13,35
Cyclin D1	overexpression/amplification	11,12,15-17
P53	mutations/overexpression	4,41-44
Retinoblastoma	decreased expression/mutation	11, 51
Cox-2	overexpressed	45,46
Wingless/Frizzled	overexpressed	2
AP-1	deregulation	1
p21	overexpressed	44,47,48
p27	loss of expression	49-52
H19	LOH	5
CENP-F	gene amplification/overexpressed	3
PAR/uPA	overexpressed	53,54
APC	LOH	55
p16	overexpressed/mutations/deletions	56-58
p14	deletions	58,59

whose expression levels may be altered in HNSCC (19-21). However, since these studies did not detect alterations in well established genes associated with HNSCC, such as EGFR and cyclin D1 (19-21), further analyses are required to confirm some of these earlier findings. It is therefore essential to develop a robust model of HNSCC and couple it with a robust analysis of alterations in gene expression between normal and neoplastic cells using gene arrays.

In this study, human keratinocytes (KCs) and cells derived from a human squamous cell carcinoma (SCC) of the tongue were grown as organotypic raft cultures and RNA isolated from these raft cultures was used to probe four gene arrays. This model of HNSCC allowed us to identify alterations in gene expression that were specific to HNSCC. The relevance of this *in vitro* model to human tumours was verified by immunohistochemical staining of selected genes in patient tumours. These data then allowed us to correlate alterations in gene expression associated with HNSCC with the biological attributes observed in neoplastic cells *in vivo*.

Materials and Methods

Cell culture. Isolation and culture of human keratinocytes (KCs) and human dermal fibroblasts (HDF) have been described (22-24). The neoplastic cell line, SCC-25, was originally derived from a human squamous cell carcinoma of the tongue (25) and its culture has been described (23).

Organotypic raft cultures and poly A$^+$ mRNA isolation. Eighteen cultures of KCs and SCC-25 cells were grown as organotypic raft cultures using previously established techniques (23,26). Total RNA from the KC and SCC-25 rafts was extracted using TRIzol (Invitrogen, Sydney, Australia) as described previously (26). Poly A$^+$ mRNA was prepared by oligo (dT) affinity chromatography27.

cDNA probe preparation and cDNA array hybridisation. Half a microgram poly A$^+$ mRNA isolated from the raft epithelia was annealed to 1 μl of CDS primer mix (Clontech, Sydney, Australia) and then labelled with [α-^{32}P] dATP (3000 Ci/mmol, Geneworks, Adelaide, Australia) as described (26). Array membranes (Human cDNA array 1.2 and Human Cancer cDNA 1.2K, Clontech) were pre-hybridised, then hybridised overnight and washed as described in the manufacturer's protocol. Membranes were exposed to a phosphoroimager screen (Molecular Dynamics, Sydney, Australia) for 24 hours. Both the human and cancer membranes (1.2k) were probed 3 times with the probes derived from the poly A$^+$ mRNA extracted from either KCs or SCC-25 cells. Probes derived from KC poly A$^+$ mRNA were probed a further 3 times on the 588 gene Human cDNA array and Cancer Array (Clontech). In total, 12 membrane arrays were probed and a total of 1895 genes were examined. Inter-membrane variability was accounted for by ensuring that the replicate probings for each cell type used different membranes. The duplication of certain genes on the various membranes resulted in the majority of genes being analysed between 3 and 12 times.

Data analysis. The intensity of hybridisation for each gene was quantified using ImageQuant (GraphPad Software, San Diego, USA [version 5.1]) and data normalised to the housekeeping genes (n=9) present on each membrane. To normalise for differences in expression for the different cell types, normalisation was also repeated between the two cell types (*i.e.* mean KC housekeeping gene expression level *vs*. mean SCC-25 housekeeping gene expression level) as previously described (26). Differences in expression between KCs and SCC-25 cell types were established using 1) fold differences and 2) a 2-tailed *t*-test. To analyse the data using fold differences, alterations in gene expression between normal KCs and the neoplastic SCC-25 cell line falling above and below the 2-fold threshold were deemed as significant. Statistical analysis using a 2-tailed *t*-test was also used to analyse the data set and significance was accepted at the $p \leq 0.05$ level. Genes showing a significant difference in expression by statistical analysis were identified and placed into broad functional groupings.

Immunohistochemistry. Deparaffinized sections of squamous cell carcinoma (SCC) of the tongue containing surrounding normal tissue were subjected to antigen retrieval and immunohistochemistry as described (26). A FRA-1 rabbit polyclonal antibody was obtained and used at a dilution of 1:100 (Santa Cruz [Monarch Medical] sc-605). Primary antibodies purchased from Sigma-aldrich (Sydney, Australia) included: cyclin D1 mouse monoclonal (C-7464, 1:30); EGFR mouse monoclonal (E-3138, 1:30); caspase 3 rabbit polyclonal (C-9598, 1:300) and PCNA mouse monoclonal (P-8825, 1:1000). Secondary antibodies used were: Biotinylated rabbit anti-mouse immunogloblins (IgG) (DAKO [Melbourne, Victoria, Australia], E0413 {1:200}) or biotinylated goat anti-rabbit IgG (DAKO, E0466 {1:1500}). Immunoreactivity was visualised using HRP-conjugated streptavidin (DAKO) and diaminobenzidine. Normal mouse IgG (DAKO, X0931) and normal rabbit IgG (Santa Cruz, sc-2027) were used as negative controls.

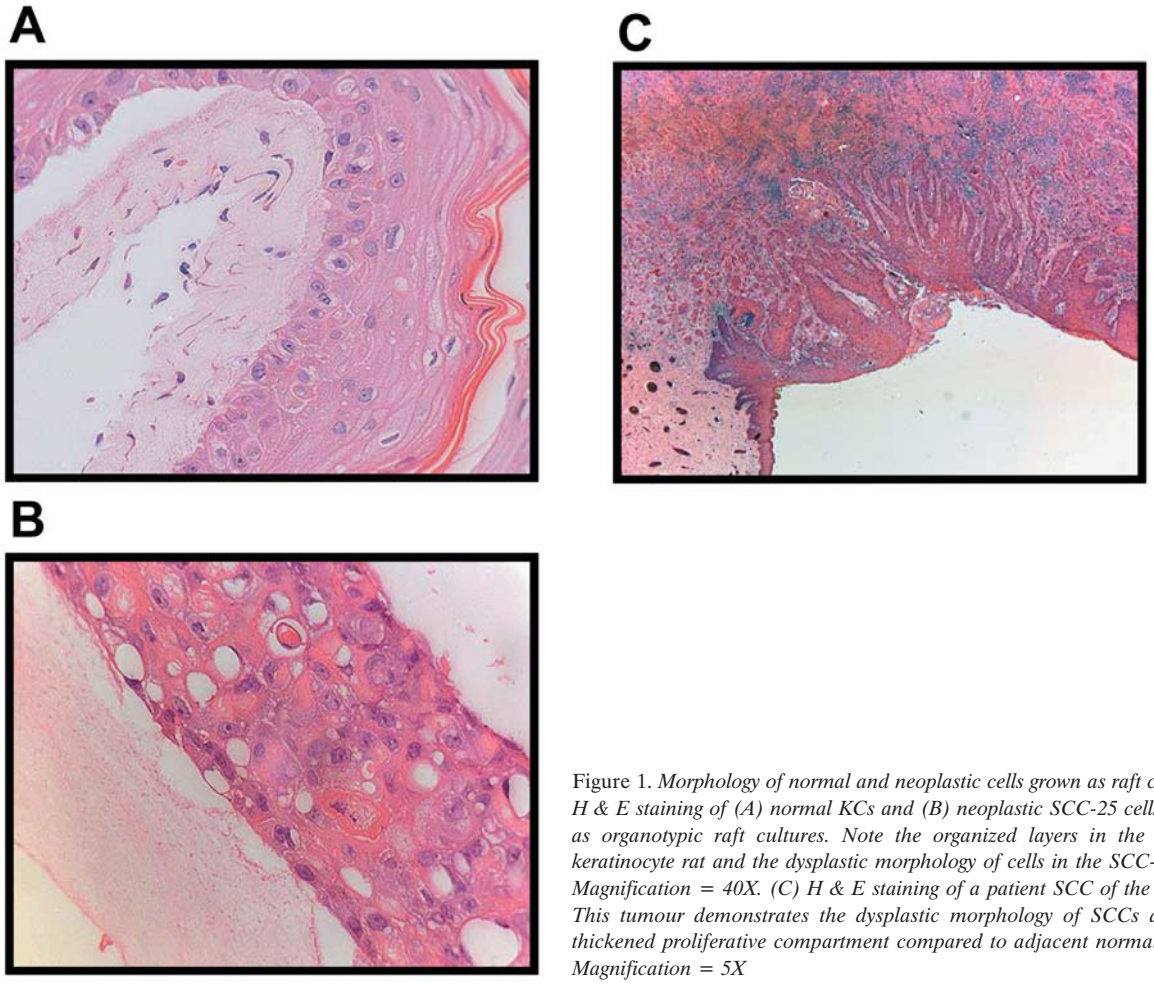

Figure 1. *Morphology of normal and neoplastic cells grown as raft cultures. H & E staining of (A) normal KCs and (B) neoplastic SCC-25 cells grown as organotypic raft cultures. Note the organized layers in the normal keratinocyte rat and the dysplastic morphology of cells in the SCC-25 raft. Magnification = 40X. (C) H & E staining of a patient SCC of the tongue. This tumour demonstrates the dysplastic morphology of SCCs and the thickened proliferative compartment compared to adjacent normal tissue. Magnification = 5X*

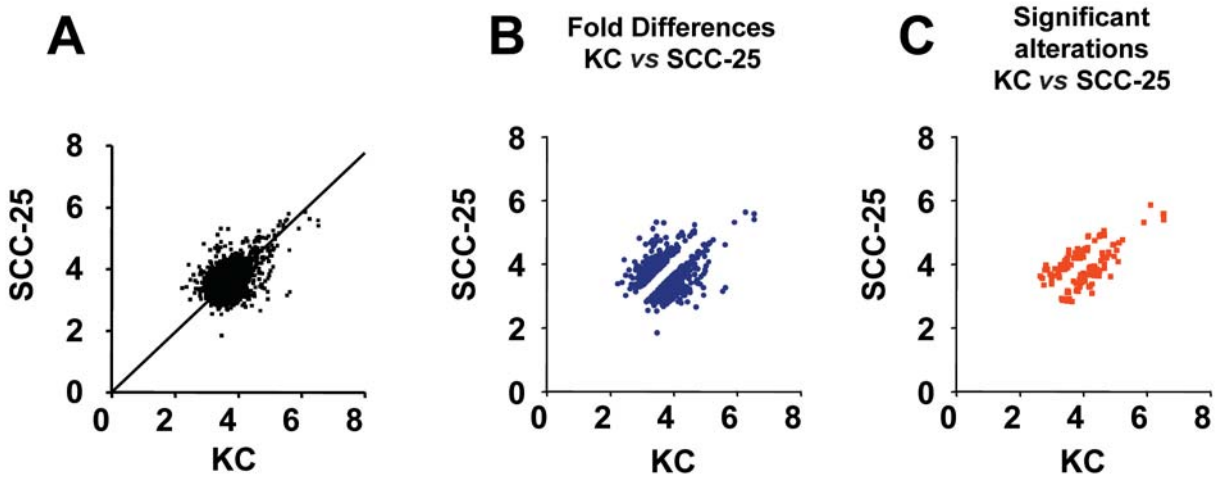

Figure 2. *Gene expression data comparing normal KC samples to neoplastic SCC-25 cells. (A) Plot of gene expression level for KCs (x axis) versus the expression level for the same gene in the SCC-25 cells (y axis). (B) Data analysis using fold alterations as the method to identify changes in gene expression between KCs vs SCC-25. 851 genes were altered 2-fold or more using this method. (C) KCs vs SCC-25 indicating the 124 genes found to be significantly different using a 2-tailed t-test.*

Table II. *Summary of genetic alterations associated with squamous cell carcinoma of the head and neck. Functional groupings of genes found to be significantly altered in their expression ($p \leq 0.05$) in HNSCC compared to normal cells by statistical analysis. Mean expression levels, the coeffecient of variance (CV), and fold alteration are shown for each gene. The genes in italics represent genes that were previously found to be common within epidermal SCC cell line (26).*

		KC		SCC-25			
	GENE	Mean	CV	Mean	CV	Alteration	
Apoptosis	*IEX-1L* anti-death protein; *PRG-1; DIF-2*	5 679.50	43.03	23 013.73	8.81	↑	4.05
	NIP3 (NIP3)	7 738.78	111.66	23 157.65	14.49	↑	2.99
	BCL-2 binding athanogene-1 (BAG-1)	10 204.70	62.22	3 443.84	61.86	↓	2.96
	caspase-3 (CASP3)	10 774.58	63.63	4 270.16	40.66	↓	2.52
Cytokines	*insulin-like* growth factor binding protein 2 (*IGFBP2*)	1 912.65	85.23	9 669.30	117.80	↑	5.06
	insulin-like growth factor binding protein 6 precursor	3 131.86	85.92	15 540.08	62.39	↑	4.96
	glia-derived neurite-promoting factor (GDNPF)	3 123.65	72.29	10 834.53	64.30	↑	3.47
	thymus-expressed chemokine precursor (TECK)	1 004.85	25.55	2 405.97	31.99	↑	2.39
	macrophage inhibitory cytokine 1 (MIC1)	18 562.53	18.00	1 230.82	70.00	↓	15.08
	thymosin beta-10 (TMSB10; THYB10); PTMB10	1 308 544.00	22.45	719 417.30	47.55	↓	1.82
Receptors/ Signal Transduction	integrin alpha 9 (ITGA9); integrin alpha-RLC	668.39	137.16	9 206.66	28.50	↑	13.77
	cadherin 4 (CDH4); *retinal cadherin precursor (R-cadherin; RCAD)*	727.55	110.84	7 034.87	98.19	↑	9.67
	putative regulatory protein TGF-beta-stimulated clone 22 homolog (TSC22)	513.87	86.87	3 657.02	47.81	↑	7.12
	protein kinase DYRK4	915.40	60.19	4 897.56	45.68	↑	5.35
	caveolin-2	2 986.64	87.95	14 388.67	68.22	↑	4.82
	CDC42 GTPase-activating protein	2 444.41	98.18	10 120.50	75.07	↑	4.14
	cadherin 6 precursor (CDH6); kidney cadherin (K-cadherin)	1 106.96	102.79	4 434.68	29.79	↑	4.01
	platelet membrane glycoprotein IA precursor (GPIA); collagen receptor	2 312.89	112.74	9 115.39	57.40	↑	3.94
	epidermal growth factor receptor (EGFR)	6 177.33	90.91	22 048.71	42.66	↑	3.57
	CD44 antigen hematopoietic form precursor (CD44H)	11 254.76	95.13	30 533.51	26.46	↑	2.71
	integrin alpha 8 (ITGA8)	1 669.40	75.07	4 389.18	19.29	↑	2.63
	prohibitin (PHB)	2 720.20	80.51	7 114.28	54.15	↑	2.62
	leukocyte adhesion glycoprotein LFA-1 alpha subunit precursor	22 922.81	80.29	55 154.74	37.96	↑	2.41
	TIS11B protein; EGF response factor 1 (ERF1)	45 772.56	23.46	80 232.05	27.16	↑	1.75
	sodium/potassium-transporting ATPase alpha 1 subunit (Na+/K+ ATPase)	30 733.86	18.41	4 306.41	30.80	↓	7.14
	phospholipase C-delta-1 (PLC-delta-1; PLCD1)	6 805.40	32.55	1 393.01	29.00	↓	4.89
	5'-AMP-activated protein kinase catalytic alpha-1 subunit	3 607.36	22.94	810.69	60.83	↓	4.45
	voltage-gated potassium channel protein KV12; HUKIV; HBK5; RBK2; NGK1	3 044.03	35.25	702.61	89.40	↓	4.33
	erythrocyte glucose transporter 1 (GLUT1)	112 458.40	12.50	27 188.01	75.84	↓	4.14
	rap1 GTPase-GDP dissociation stimulator 1	5 911.12	23.08	1 477.39	79.36	↓	4.00
	voltage-gated potassium channel protein KV11; HUKI; HBK1	7 670.74	14.35	1 953.12	36.86	↓	3.93
	extracellular signal-regulated kinase 3 (ERK3); MAP kinase 3 (MAPK3)	27 066.08	82.06	6 939.40	39.19	↓	3.90
	recoverin; cancer-associated retinopathy protein (CAR protein)	11 369.07	24.75	3 014.37	56.50	↓	3.77
	casein kinase I gamma 2 (CKI-gamma 2)	7 812.96	35.46	2 182.44	45.09	↓	3.58
	Summary of genetic alterations associated with squamous cell carcinoma of the head and neck.						
	interferon- induced protein P78	55 070.87	5.94	16 260.60	108.89	↓	3.39
	S100 calcium-binding protein A1; S-100 protein alpha chain	20 239.95	38.11	5 997.33	39.76	↓	3.37
	metabotropic glutamate receptor 1 precursor (GRM1; MGLUR1)	2 254.14	30.19	741.50	44.81	↓	3.04
	transforming protein rhoA H12 (RHO12; ARH12; ARHA)	63 177.36	48.75	20 906.69	49.96	↓	3.02
	interferon-inducible protein 9-27	172 918.90	21.74	57 936.25	20.04	↓	2.98
	tuberin; tuberous sclerosis 2 protein (TSC2)	13 152.23	16.17	4 636.84	32.69	↓	2.84
	T-cell surface glycoprotein E2 precursor; CD99 antigen; MIC2 protein	9 533.24	28.78	3 363.10	63.59	↓	2.83

Table II. *Continued.*

	GENE	KC Mean	KC CV	SCC-25 Mean	SCC-25 CV	Alteration	
	alpha1 catenin (CTNNA1); cadherin-associated protein; alpha E-catenin	23 102.21	45.33	8 223.05	52.09	↓	2.81
Receptors/ Signal Transduction	NCK melanoma cytoplasmic src homolog (HSNCK)	7 926.45	46.23	2 889.78	85.78	↓	2.74
	guanylate cyclase soluble beta-1 subunit; guanylate cyclase 70-kDa subunit	17 852.74	16.62	6 639.03	37.89	↓	2.69
	Herpes virus entry protein C (HVEC)	13 000.96	16.60	4 934.74	35.40	↓	2.63
	serine/threonine protein phosphatase 2B catalytic subunit alpha isoform	9 367.08	35.96	3 588.09	33.93	↓	2.61
	ephrin type-A receptor 1 precursor; tyrosine-protein kinase receptor eph	62 203.92	23.78	23 858.49	9.48	↓	2.61
	MAP kinase-activated protein kinase kinase 2 (MAP kinase kinase 2; MAPKK 2)	9 250.12	39.01	3 589.79	65.37	↓	2.58
	ADP/ATP carrier protein	21 688.53	13.27	8 756.54	43.49	↓	2.48
	lnk adaptor protein	3 138.96	34.26	1 267.64	25.36	↓	2.48
	ephrin type-A receptor 2 precursor; epithelial cell kinase (ECK)	7 760.69	59.94	3 403.45	49.99	↓	2.28
	RalB GTP-binding protein	5 838.49	21.12	2 613.24	45.46	↓	2.23
	MHC class I truncated HLA G lymphocyte antigen	85 864.18	24.99	40 943.96	15.47	↓	2.10
	cAMP-dependent protein kinase I alpha regulatory subunit	8 312.83	32.68	4 677.51	36.61	↓	1.78
	melanoma antigen P15	1 948.05	76.61	7 683.31	28.52	↑	3.94
	collagen 8 alpha 1 subunit (COL8A1)	581.93	59.01	2 203.99	69.98	↑	3.79
	fibronectin precursor (FN)	3 101.09	67.53	11 081.27	25.03	↑	3.57
	matrix metalloproteinase 8 (MMP8);PMNL collagenase	995.05	122.01	3 217.88	64.63	↑	3.23
	metalloproteinase inhibitor 3 precursor	5 138.25	65.55	15 599.20	32.61	↑	3.04
Extracellular Matrix/ Proteases Migration	alpha-2-macroglobulin receptor-associated protein precursor (alpha-2-MRAP; A2MRAP)	5 974.84	67.44	15 991.84	37.86	↑	2.68
	proteasome activator HPA28 subunit beta	9 000.43	17.66	21 780.32	20.59	↑	2.42
	tumor suppressor maspin; protease inhibitor 5 (PI5)	8 008.10	26.07	15 250.63	22.28	↑	1.90
	paxillin	10 165.03	36.02	16 637.31	3.99	↑	1.64
	calgranulin B; migration inhibitory factor-related protein 14 (MRP14)	3 378 265.00	43.53	254 133.40	31.71	↓	13.29
	calgranulin A; migration inhibitory factor-related protein 8 (MRP8);	3 339 051.00	56.68	383 232.00	26.21	↓	8.71
	placental plasminogen activator inhibitor 2 (PAI-2)	15 079.34	60.58	2 029.71	60.27	↓	7.43
	calpain 1 large (catalytic) subunit	32 933.60	14.12	6 375.37	23.46	↓	5.17
	leukocyte elastase inhibitor (LEI)	45 894.11	25.17	10 340.41	34.07	↓	4.44
	calpain 2 large (catalytic) subunit	19 795.18	43.24	4 650.85	32.90	↓	4.26
	tastin	3 237.12	39.44	803.17	22.33	↓	4.03
	calcium-dependent protease small (regulatory) subunit	49 714.98	17.34	15 643.79	41.46	↓	3.18
	PRSM1 metallopeptidase	16 365.65	22.18	6 716.10	38.47	↓	2.44
	neutrophil gelatinase-associated lipocalin precursor (NGAL)	91 368.77	9.82	41 746.77	51.42	↓	2.19
	glutathione peroxidase-gastrointestinal (GSHPX-GI)	3 495.43	13.49	27 656.49	12.96	↑	7.91
	ribonuclease 6 precursor	1 290.26	95.20	7 078.33	41.51	↑	5.49
	thioredoxin peroxidase 2 (TDPX2); proliferation-associated gene (PAG)	13 967.78	95.84	72 236.20	25.34	↑	5.17
	heat shock 90-kDa protein A (HSP90A; HSPCA); HSP86	44 854.07	54.81	114 477.90	10.93	↑	2.55
	HHR23A; UV excision repair protein protein RAD23A	13 782.21	49.88	33 907.97	59.58	↑	2.46
Metabolism	cytoplasmic dynein light chain 1 (HDLC1)	32 202.47	59.22	75 704.91	8.82	↑	2.35
	dopamine beta-hydroxylase (DBH); dopamine-beta-monooxygenase precursor	1 756.12	56.91	3 825.75	20.16	↑	2.18
	(2'-5')oligoadenylate synthetase 1 ((2-5')oligo(A) synthetase 1)	16 064.47	16.44	1 935.91	23.85	↓	8.30
	dipeptidyl-peptidase I precursor (DPP-I)	45 625.61	20.68	7 406.96	8.76	↓	6.16
	acyl-CoA-binding protein (ACBP)	93 830.23	40.93	17 931.75	30.57	↓	5.23
	S100 calcium-binding protein A7; psoriasin	808 239.70	5.02	206 406.60	10.54	↓	3.92
	placental calcium-binding protein; S100 calcium-binding protein A4	35 973.10	22.03	12 875.54	26.36	↓	2.79

Table II. *Continued.*

	GENE	KC Mean	KC CV	SCC-25 Mean	SCC-25 CV	Alteration	
Metabolism	heme oxygenase 1 (HO1); HSOXYGR	3 500.90	10.45	1 286.64	28.13	↓	2.72
	leptin precursor; obesity factor; obese protein	3 379.75	10.04	1 317.61	86.50	↓	2.57
	dimethylaniline monooxygenase (N-oxide forming) 1	2 068.77	10.20	831.05	84.49	↓	2.49
	lecithin-cholesterol acyltransferase (LCAT)	3 242.39	21.10	1 433.63	34.80	↓	2.26
	26S protease regulatory subunit 6A; TAT-binding protein 1 (TBP1)	15 315.96	7.80	7 689.57	17.77	↓	1.99
	fte-1; 40S ribosomal protein S3A (RPS3A)	39 885.50	6.87	22 554.99	42.77	↓	1.77
	glutathione synthetase (GSH synthetase; GSH-S)	4 886.42	27.11	2 914.63	27.80	↓	1.68
Structural	BIGH3	9 056.75	75.31	27 484.21	63.90	↑	3.03
	type II cytoskeletal 11 keratin (KRT11); cytokeratin 1 (K1; CK1)	7 737.68	54.17	18 795.63	6.82	↑	2.43
	type I cytoskeletal 19 keratin; cytokeratin 19 (K19; CK19)	125 810.30	40.04	12 021.23	82.65	↓	10.47
Proliferation/ Cell Cycle	*cyclin-dependent kinase 5 activator isoform (CDK5 activator; NCK5AI)*	632.40	124.67	6 721.54	80.48	↑	10.63
	5T4 oncofetal antigen precursor	3 471.05	61.08	20 038.32	17.14	↑	5.77
	p21-activated kinase gamma (PAK-gamma; PAK2); PAK65; S6/H4 kinase	2 651.97	82.15	12 844.71	64.48	↑	4.84
	G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	6 071.00	93.44	20 309.06	42.73	↑	3.35
	cyclin-dependent kinase regulatory subunit 1 (CKS1)	1 586.30	54.99	4 594.90	30.10	↑	2.90
	retinoblastoma-like protein 2 (RBL2; RB2)	2 477.77	99.60	6 724.69	61.74	↑	2.71
	proliferating cell nucleolar antigen P120; NOL1	3 258.26	95.06	7 957.70	23.60	↑	2.44
	cell cycle progression 2 protein (CPR2)	4 016.14	38.71	9 557.52	24.74	↑	2.38
	cyclin K	9 459.33	29.81	18 617.98	19.19	↑	1.97
	cyclin G-associated kinase (GAK)	6 518.63	17.92	1 676.15	66.18	↓	3.89
	CDC7-related kinase	7 493.30	26.38	2 658.83	75.38	↓	2.82
	RCL growth-related c-myc-responsive gene	16 921.22	16.90	8 323.71	17.60	↓	2.03
Transcription factors/DNA	DNA-binding protein UEV-1; UBE2V	439.34	55.40	4 226.71	45.33	↑	9.62
	fos-related antigen (FRA1)	14 518.34	131.03	78 800.77	38.35	↑	5.43
	fau	34 670.21	39.59	84 638.54	32.39	↑	2.44
	DNA-binding protein A	13 348.28	36.63	26 836.90	12.20	↑	2.01
	DNA-binding protein inhibitor ID-1; Id-1H	71 513.17	49.37	4 116.17	46.26	↓	17.37
	growth arrest & DNA-damage-inducible protein (GADD45)	22 499.81	76.50	4 350.87	85.24	↓	5.17
	nucleobindin precursor (NUC)	22 828.59	50.34	5 645.73	15.47	↓	4.04
	high mobility group protein (HMG-I)	133 514.40	16.44	47 510.05	31.33	↓	2.81
	R kappa B DNA-binding protein	10 304.65	33.63	4 711.95	21.71	↓	2.19
	replication protein A 14-kDa subunit (RP-A) (RF-A); replication factor A protein 3	954.23	18.94	5 296.54	21.14	↓	1.88
Miscellaneous	major prion protein precursor (PRP); PRP27-30; PRP33-35C; ASCR	6 630.37	20.38	30 719.80	16.56	↑	4.63
	KIAA0324	1 663.97	53.13	6 081.51	35.46	↑	3.65
	B94 protein	18 718.83	25.54	2 394.16	86.98	↓	7.82
	paraneoplastic encephalomyelitis antigen HUD; HU-antigen D	4 435.87	43.83	665.99	88.33	↓	6.66
	HEM45	9 205.02	18.85	2 690.17	95.08	↓	3.42
	C-reactive protein precursor	13 715.81	21.84	4 849.96	48.14	↓	2.83
	cathepsin D precursor (CTSD)	40 368.14	28.59	16 934.82	33.00	↓	2.38

Results

Raft cultures resemble the architecture of normal and neoplastic cells in vivo. The normal keratinocytes and the neoplastic SCC-25 cells were grown as raft cultures in an attempt to recapitulate the normal architecture of these cells *in vivo* in the absence of contaminating cell types (28-31). As can be seen in Figure 1A, the normal KCs form a highly organised multi-layered structure similar to that of normal epithelial tissue. These cells form cuboidal basal cells, appear more flattened within the suprabasal layers and form a clear stratum corneum. In contrast, the neoplastic SCC-25 cells (originally isolated from a SCC of the tongue (25)) form poorly defined layers, with an atypical basal layer, poorly differentiated suprabasal layers and evidence of dysplasia throughout (Figure 1B). A similarly dysplastic appearance is seen in tongue lesions *in vivo* (Figure 1C). The histological data presented in Figure 1, coupled with our earlier immunohistochemical analyses of raft cultures (26), demonstrate that the culture of KCs and SCC-25s in organotypic raft cultures are a robust source of cells that have characteristics of normal or transformed keratinocytes.

Gene expression levels are altered during HNSCC formation. In order to identify alterations in gene expression associated with the neoplastic phenotype, normal KC gene expression levels were compared to the expression levels of genes from the neoplastic SCC-25 cells using gene arrays. The neoplastic cells express a number of genes whose mRNA expression appears to be altered compared to normal cells (Figure 2A).

In order to identify potential gene alterations associated with normal and neoplastic cells, fold differences with a significance inferred by a ≥ 2-fold difference in gene expression was initially used. This arbitrary approach indicated that the expression levels of 851 (44.9%) genes were considered different between KCs and SCC-25 (Figure 2B). In contrast, the same data set, analysed using a more robust, 2-tailed t-test, indicated that the expression level of only 124 (6.5%) genes were significantly altered between the KC and SCC-25 cells (Figure 2C and Table II). These 124 significantly different gene alterations were further divided into a variety of different functional groups (Table II). These include genes involved in apoptosis, cell cycle regulation, migration and metabolism. Further, comparing these gene alterations to gene alterations previously identified in epidermal SCCs (26) (ESCC), indicated 36 gene alterations were common to both HNSCC and ESCC. These data suggest that of the 124 altered genes, 36 gene alterations may be generically associated with SCC formation (Table II, italics) and the majority (88 genes [71%]) were HNSCC-specific.

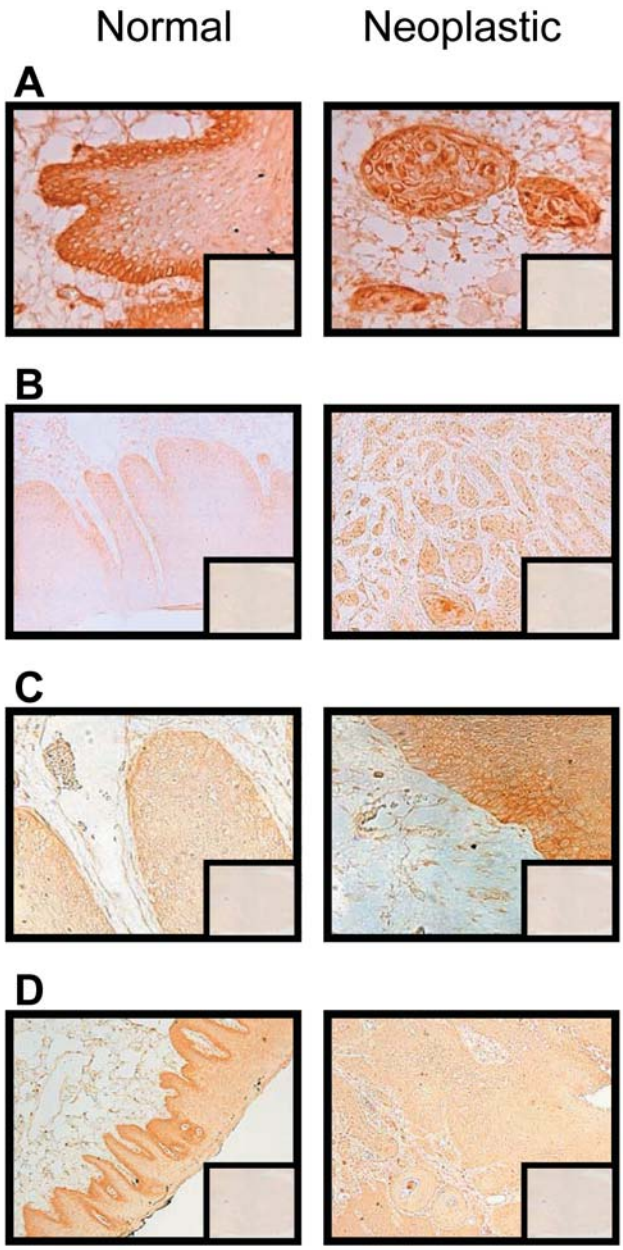

Figure 3. *Immunohistochemical confirmation of differences between normal and neoplastic epithelial cells. A squamous cell carcinoma of he tongue from a patient with adjoining normal tissue was stained for (A) FRA-1, (B) cyclin D1, (C) EGFR, (D) Caspase 3. Inset = control staining. Magnification = 10X (A, B). Magnification = 40X (C, D).*

Alterations in gene expression are reflected in protein expression in vivo. To validate the gene expression data, patient tumours were examined using immunohistochemical staining for four genes representing different functional classes which were found to be altered between KCs and SCC-25s using gene array analysis (Figure 3). Squamous cell

carcinoma of the tongue with histologically 'normal' tissue adjacent to the lesion was used to avoid inter-slide variability (Figure 1B). All the immunohistochemical staining showed the same qualitative trend as the gene array data. Fos related factor-1 (FRA-1) is a member of the oncogenic AP-1 transcription factor family (32,33). FRA-1 mRNA expression was shown to be significantly increased in the neoplastic SCC-25 cells when the gene array data was analysed using a t-test (5.43 fold). Immunoreactive FRA-1 protein was high in the basal layer of the normal epithelium, whereas in the SCC lesion, expression was high throughout the tumour mass (Figure 3A). Cyclin D1 is an essential cell cycle regulator that promotes proliferation and has been previously shown to be deregulated in HNSCCs (11,15,16). This deregulation is also observed in our analysis where both mRNA (3.35 fold) and protein are increased in the tumour sample (Figure 3B). Similarly, we found an increase in both mRNA expression and protein expression of the EGFR (Figure 3C), consistent with many reports in HNSCC (10,13,34,35). Caspase 3 is involved in the activation of the apoptotic program (36,37). Caspase 3 expression is reduced in tumour cells using both gene array analysis (2.52 fold) and immnohistochemistry (Figure 3D). These data demonstrate that our gene array analysis provides a robust representation of alterations that occur in patient tumours. Moreover, since cyclin D1 and EGFR over-expression are established markers of HNSCC, these data also strongly validate our model. Although only one cancer cell line was used in our gene array analysis the observation that the gene expression changes noted were also present in patient tumours provides definitive proof of the validity of this strategy.

Discussion

Earlier studies of alterations in gene expression occurring in HNSCC used RNA from patient tumours (19-21). Since patient tumours invariably contain non-tumour tissue, it can be difficult to definitively identify alterations in gene expression that are tumour cell-specific. In the present study, we have utilised an *in vitro* model that recapitulates the *in vivo* structure and function of normal and neoplastic epithelia (23,26). The use of an organotypic raft culture system allowed us to mimic the spatial and temporal relationships of keratinocytes in a similar manner to that of "native" epithelia (whether normal or neoplastic in origin) (23,26) (Figure 1). More importantly, these rafts gave us access to RNA from normal or neoplastic keratinocytes in the absence of contaminating non-epithelial cell types (28-31,38). The rafts, therefore, provided a robust and relevant model in which to examine alterations in gene expression associated with HNSCC development.

Whilst earlier gene array studies have utilised fold alterations in gene expression as their criteria for significance, this approach had some difficulty discriminating between false-positives and false-negatives. For this reason there is a trend amongst current gene array/microarray studies to use more robust statistical methods, usually on repeated probings of the same samples, or single probings on multiple samples to analyse their data. This form of analysis has the potential to be more robust and in the present study we clearly show that analysing gene expression differences purely on fold changes revealed that approximately 44% of the genes analysed were altered. Such a large number of genes ($\sim$850) whose expression is altered in HNSCC cells would undoubtedly contain false-positives/negatives and would be difficult to pursue with respect to their functional contribution to neoplasia. However, the application of more rigorous statistical analyses (*i.e.* 2-tailed *t*-test) to the same data set reduced the number of genes whose expression was significantly altered to 6.5% for HNSCC cells (SCC-25) compared to the normal KC cells (Figure 2C). Thus, most genes whose expression were either increased or decreased by two-fold did not reach significance when analysed more stringently at a 95% confidence interval. These data suggest that repeated sample probings allows for a more robust identification of altered gene expression.

Although our study examined the gene expression alterations of only one HNSCC cell line (SCC-25), we validated our gene array data by selecting four genes from different functional groups and confirming their altered expression in patient tumours by immunohistochemistry. Cyclin D1, EGFR, Caspase 3 and FRA-1 were selected to examine expression at a protein level in patient tumours since they may contribute to the deregulated growth, increased lifespan and invasiveness of the tumours. In all the instances in which immunohistochemical determination was made in patient tumours, the data was similar to that obtained from the gene array data. Epidermal growth factor receptor and cyclin D1 have been reported to be deregulated in a majority of HNSCCs, with incidences as high as 62% and 68%, respectively, in some studies (12,13). These data strongly validate the use of organotypic raft cultures as a model of HNSCC *in situ*. Since this study was restricted to the analysis of 2000 genes, it would be expected that a more comprehensive study of the entire human genome, and perhaps additional cell lines, would identify other potentially important gene alterations associated with this disease.

It is important to consider that of the genes exhibiting altered expression in the HNSCC cells, some alterations will be specific to cancer (*e.g.* generic cancer associated genes) whilst some will be specifically associated with HNSCC. In this regard, we previously reported on alterations in the

expression of 37 genes that could be considered SCC-specific (epidermal and head and neck SCC) (26). In the present study, we show that in, addition to these 37 SCC-specific genes, we have identified a further 88 genes that are specifically altered in HNSCC (*i.e.* HNSCC-specific). These putative HNSCC-specific alterations are of considerable interest since they may provide insight into mechanisms of HNSCC development. For instance, the formation of head and neck squamous cell carcinomas is associated with excessive smoking and alcohol consumption (4-6). Consequently, some or all of these 88 genes may relate specifically to alcohol or tobacco-induced carcinogenesis.

In this regard it was of particular interest to note that genes that contribute to detoxification reactions were selectively altered in the HNSCCs (*e.g.* glutathione synthetase, thioredoxin peroxidase and glutathione peroxidase 2 [Table II]). The disruption of glutathione synthetase and thioredoxin peroxidase found in this study may be pertinent to the etiology of HNSCC. Both these genes are associated with the detoxification of harmful reactive oxygen species (ROS) (39,40). Head and neck SCC formation is associated with excessive smoking (4-6) and the toxins contained in cigarette smoke are known to be metabolised *via* reactive intermediates. Hence, cells within the oral cavity may be exposed to these reactive intermediates resulting in oxidative damage or apoptosis. Therefore, if detoxification of these harmful by-products is compromised, it is possible that the accumulation of ROS within these cells may contribute to HNSCC development. The marked induction of the antioxidant enzyme, glutathione peroxidase 2 (GPx-2), was of particular interest since it was also reported to be induced in epidermal SCCs (26). This finding suggests that GPx-2 may be important in the development of multiple tumour types and suggests that its alteration is not likely to be due solely to stimuli involved in HNSCC development.

An exhaustive analysis of the 88 HNSCC-specific genes that display altered expression in HNSCC cells is not possible in this manuscript. However, in addition to the genes associated with detoxification, it is interesting to note that several other genes identified in this study may contribute to the phenotype observed in HNSCC. For example, the increased expression of the cyclin D1 gene in the neoplastic cells would be expected to deregulate the activity of the cell cycle protein, E2F (19,22). Interestingly, E2F activity and E2F1 expression are increased in HNSCC cells (9,22). The result of this deregulation would be growth inhibitor insensitivity and constitutive proliferation. Furthermore, the decrease in capase-3 mRNA expression in cancer cells may allow these cells to evade apoptosis. Caspase-3 is a downstream effector of apoptotic stimuli (*e.g.* ROS) and as such would be a mediator of the apoptotic response to physical and chemical insults (*e.g.* tobacco smoke). However, in the absence of a functional caspase-3 protein, such a response would be attenuated allowing cells containing mutated DNA to avoid apoptosis. Together, the alterations in caspase-3, detoxification enzymes and cyclin D1 observed in HNSCC may serve to deregulate the growth, increase the lifespan and enhance ROS-mediated damage in HNSCC cells.

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