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SHORT REPORT

Distinct pattern of expression of differentiation and growth-related genes in squamous cell carcinomas of the head and neck revealed by the use of laser capture microdissection and cDNA arrays

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Although risk factors for squamous cell carcinomas of the head and neck (HNSCC) are well recognized, very little is known about the molecular mechanisms responsible for this malignancy. Furthermore, the ability to investigate gene expression profiles at different stages of tumor progression is usually limited by the remarkable heterogeneity of these neoplastic lesions. Here, we show the successful use of laser capture microdissection (LCM) to procure specific cell populations. The 5000 cells from representative sets of HNSCC and their matching normal tissues are sufficient to extract RNA of high integrity for the synthesis of labeled amplified cDNA probes which can then be hybridized to these membranes arrayed with known human cancer-related cDNAs. Furthermore, when compared to normal tissues, we demonstrate a consistent decrease in expression of differentiation markers such as cytokeratins, and an increase in the expression of a number of signal transducing and cell cycle regulatory molecules, as well as growth and angiogenic factors and tissue degrading proteases. Unexpectedly, we also found that most HNSCC overexpress members of the *wnt* and *notch* growth and differentiation regulatory system, thus suggesting that the *wnt* and *notch* pathways may contribute in squamous cell carcinogenesis. This experimental approach may facilitate the identification candidate markers for the early detection of preneoplastic lesions, as well as novel targets for pharmacological intervention in this disease. *Oncogene* (2000) **19,** 3220–3224.

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Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer in developed countries, and of the 44 000 annual cases reported in the United States, approximately 11 000 will result in an unfavorable outcome (Landis *et al*., 1999; Parkin *et al*., 1999). In spite of its high incidence, the molecular mechanisms of this disease remain poorly understood. However, the recently gained knowledge of normal and aberrant function of oncogenes and tumor suppressor genes has provided unique opportunities to understand, and ultimately to control, the processes leading to malignancy. Thus, the identification of the molecular and genetic events involved in each step of tumor progression may be central to understand HNSCC, and for the development of diagnostic markers and novel treatment strategies.

Although HNSCC is thought to result from the progressive accumulation of genetic lesions leading to malignancy (Mao *et al*., 1998) the precise nature of the affected molecules is still largely unknown. The recent development of several high throughput, hybridization-based methods utilizing cDNAs arrayed on nylon membranes and glass slides allows the analysis of hundreds of genes simultaneously, and thus provides a unique opportunity to identify genes expressed in normal and tumor tissues, as well as to analyse gene expression profiles in tumor progression. However, an accurate procurement of specific cell types for RNA isolation is a critical step influencing the validity of this analysis. In this regard, a novel technique of Laser Capture Microdissection (LCM) developed at the Laboratory of Pathology (National Cancer Institute), enables the procurement of pure cell populations from frozen human tissue sections (Emmert-Buck *et al*., 1996; Simone *et al*., 1998), a key consideration as many tumors, including HNSCC, are heterogeneous, and include areas of connective tissues, blood vessels and even inflammatory cells that infiltrate into the tumor mass. Most importantly, microdissection allows analysis of gene expression in specific cell populations as it exists *in situ*. In this study, we have used LCM to procure specific cell populations from a representative set of tumors and their matching normal tissues to explore the feasibility of establishing a pattern of expression of cancer-related genes for HNSCC.

Extraction of RNA from HNSCC and normal epithelial cells procured by laser capture microdissection

Clinical characteristics of the human biopsies from HNSCC patients who had undergone surgery and were chosen for the study are indicated in Table 1. The

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Table 1 Clinical characteristics of lesions from patients with HNSCC

Case	*Origin*	*Lesion*	*Pathology*
WSU 1	Tongue	Hyperplasia	Severe hyperplasia
WSU 51	Laryngeal	Carcinoma	High clinical grade Invasive and poorly differentiated
WSU 58	Pharyngeal	Carcinoma	Mild to moderate dysplasia, moderate to well differentiated and invasive in places
WSU 62	Tongue	Carcinoma	Invasive, moderate to well differentiated
WSU 63	Tongue	Carcinoma	Moderate to poorly differentiated and invasive

HNSCC lesions upon biopsy were analysed for clinical classification. The five tissue sets (WSU 1, 51, 58, 62, 63) were biopsies from patients previously confirmed to have neoplastic lesions of the head and neck. Anatomical site and severity of the lesions are indicated

anatomical sites of these lesions are representative of the most frequent HNSCC sites, and include the tongue (WSU 1, 62 and 63), larynx (WSU 51), and pharynx (WSU 58). The pathology that was provided with the tissues indicated that all lesions except WSU 1, were carcinomas, either poorly (WSU 51, 63) or moderate to well differentiated (WSU 58, 62). All tumors were invasive. Tissue WSU 1 was confirmed as hyperplasia. Corresponding normal tissue, from the same anatomical site and patient as the lesion, was part of the tissue set and consisted of normal epithelium. We considered these five tissue sets to be representative of HNSCC, and thus suitable for assessing the feasibility of using LCM for the detailed analysis of gene expression in these cancer lesions. Before proceeding, the histopathology was confirmed by a board certified pathologist. Microscopic visualization of representative frozen tissue sections (8 μm thickness), stained with hematoxylin-eosin is shown in Figure 1A (a–d).

As shown in Figure 1B, the use of LCM enables the procurement of a pure population of squamous cells (>95% purity) that can be used to extract RNA. Quality assessment of the RNA extracted from each tissue was performed by RT–PCR of GAPDH. As demonstrated in Figure 1C, RNA from two representative tissue sets (WSU 62, 63) was of sufficient quality to be reverse transcribed and amplified using specific primers for GAPDH, which generated a 600 bp product. Similar results were obtained with the remaining three tissue sets (data not shown), thus supporting that LCM preserves the integrity of the RNA extracted from HNSCC cells.

Hybridization and gene expression

Total RNA was reverse-transcribed using Superscript Reverse Transcriptase (Life Technologies) and cDNAs synthesized for each sample were assessed for integrity. In all cases, the average size of the reverse-transcribed messages was approximately 500 bp (range 300–800 bp, data not shown), which is similar to that achieved for the construction of highly representative cDNA libraries (Peterson *et al*., 1998). cDNAs corresponding to normal and tumor tissue from the same set were simultaneously amplified and labeled with α-^{33}P dCTP. Labeled amplified cDNAs (AcDNAs) were then used to hybridize human cancer cDNA arrays (CLONTECH) containing 200–500 bp DNA fragments, in duplicates, for 588 known human cancer and nine housekeeping genes (http://www.clontech.com/atlas/genelists). In preliminary experiments, this procedure resulted in a highly reproducible pattern of gene expression when using the same RNA preparation and independently AcDNAs. Membranes hybridized with complex cDNA probes from a representative tissue set are shown in Figure 2A and illustrates the comparative differences in expression of genes belonging to different functional groups in both normal (upper panel) and tumor tissue (lower panel) from the same patient.

Genes differentially expressed

Examples of genes differentially expressed are shown in Figure 2B and include the cytokeratins (a) and those genes belonging to the MAPK (mitogen-activated protein kinase) (b) and *wnt* (wingless) (c) signaling pathways. The amount of radiolabeled probe hybridized to each arrayed cDNA was quantified using PhosphorImaging, and normalized by that hybridized to the housekeeping genes (GAPDH). In preliminary experiments, we found that under these experimental conditions differences of $\geqslant$twofold were reproducible, and the radioactivity within the linear range of detection. To simplify the analysis, the main functional groups of genes assessed to be differentially expressed $\geqslant$twofold in at least three of the four tumor sets when comparing each cancerous epithelium to their corresponding normal tissue were considered of likely biological significance, and are listed in Table 2. They include genes involved in the control of cell growth and differentiation, angiogenesis, apoptosis, cell cycle, and signaling, most of which have not been previously implicated in HNSCC when using other analytical approaches. These data indicate that complex AcDNA probes labeled with α-^{33}P dCTP can be successfully synthesized from small amounts of total RNA for their use in comparative hybridization studies of cancer genes expressed in HNSCC. Furthermore, these findings have helped identify a number of new candidate genes, which might play an unexpected role in squamous carcinogenesis.

One of the most remarkable changes was the general decrease in the expression of cytokeratins (2E, 2P, 6A-F, 7, 13, 14, 15, 17, 18, 19), which were readily detected in normal tissue but nearly absent in the cancer cell population (2–20-fold reduction), most likely reflecting the loss of differentiation in tumor cells. On the other hand, we observed a clear increase in the levels of *cyclin D1* (2–3-fold), as previously reported (Bartkova *et al*., 1995), and those of metalloproteases (*MMP-7*, *MMP-10*, *MMP-14*), which reflects the highly invasive behavior of this tumor type. Furthermore, we observed a remarkable increase in the levels of many growth and angiogenic factors including *TGFα*, *TGFβ*, *EGF Cripto protein*, *PDGF A chain* and *B chain* (*c-Sis*), different *FGF* isoforms, *HGF*, and *VEGF-C*. This supports the conclusion that this tumor type secretes factors that are likely to induce epithelial cell growth in an autocrine fashion in addition to promoting the growth of stromal cells and the process of neovascularization (Desai and Libutti, 1999). Furthermore, these tumors also overexpress several proapoptotic molecules, including

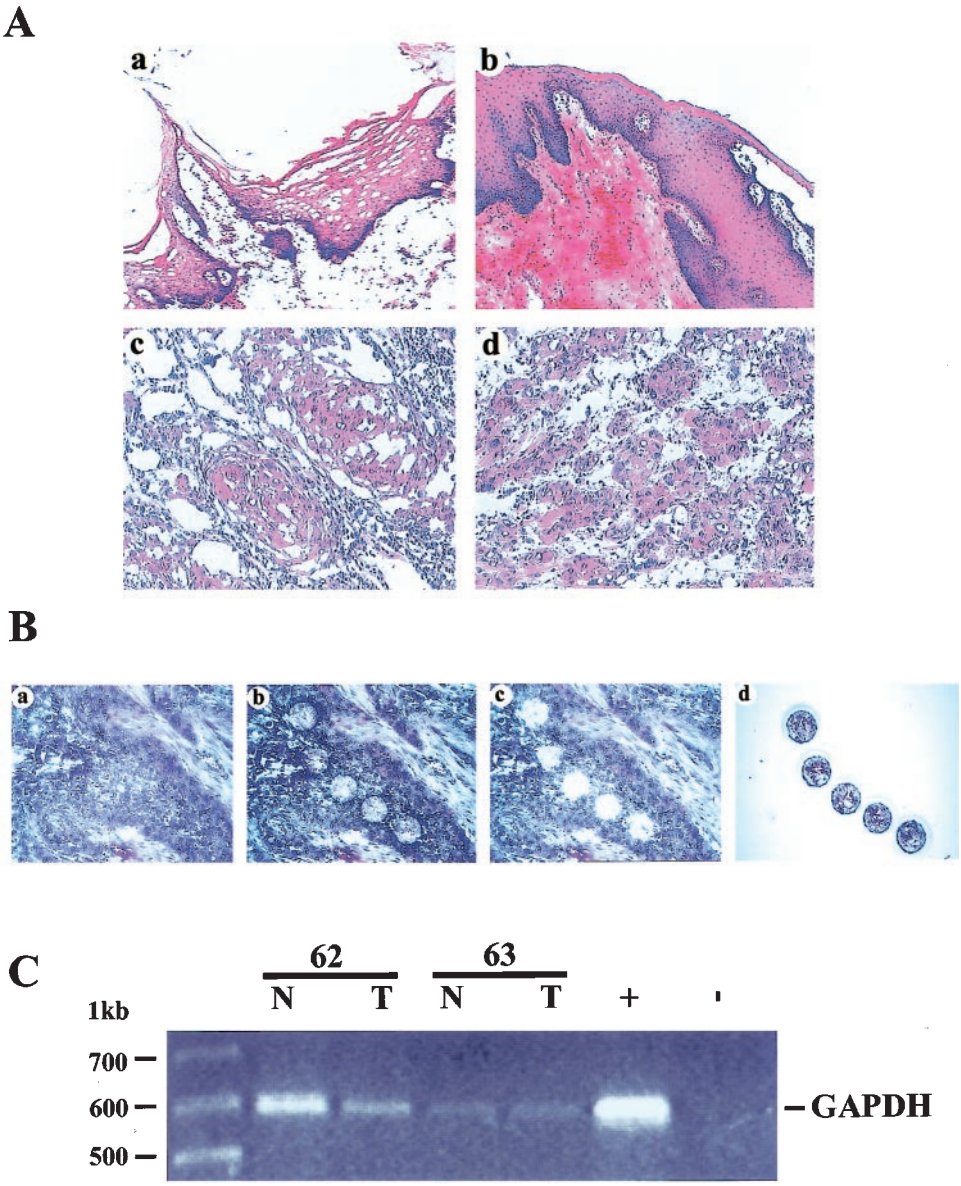

Figure 1 (**A**) Histopathological features of HNSCC. Tissue sets, comprising of both normal and tumor from the same HNSCC patient, were snap frozen and 8 μm sections were stained with H&E. Histopathological features of progression of HNSCC from normal to carcinoma are illustrated. Representative normal squamous epithelium (**a**) from head and neck region shows an orderly maturation from deep to superficial cell layers marked by progressive flattening of the cells and nuclei (magnification $\times 200$). Hyperplasia (**b**) is characterized by increased layers of epithelial cells throughout the lower third of the epithelium, normal maturation, and intact basement membrane (magnification $\times 200$). Well-differentiated invasive squamous cell carcinoma (**c**) shows infiltration of the underlying stroma, loss of normal architecture, occasional keratin pearls. There is mild nuclear pleomorphism and increased numbers of mitotic figures (magnification $\times 500$). Moderate to poorly-differentiated invasive carcinoma (**d**) shows infiltration of the underlying stroma with sheets of cancer cells showing marked nuclear pleomorphism and hyperchromasia. There is notable absence of keratin pearls (magnification $\times 500$). (**B**) For LCM procurement, an area of tumor, containing cells of interest are visualized (**a**) and targeted for capture with a 30 μm diameter laser beam (**b**). The caps containing the captured cells are lifted off the tissue section (**c**), and the homogeneity of these cells is confirmed under a light microscope (**d**) prior to processing for RNA extraction. Each laser beam procures 3–7 cells (magnification $\times 500$). (**C**) Total RNA was extracted from microdissected tissues (approximately 5000 cells) using guanidium isothiocyanate (GITC) and phenol/chloroform extractions, isopropanol precipitation, and treatment with DNAse I in the presence of RNAse inhibitors. The integrity of the RNA was assessed by amplifying GAPDH by RT–PCR, using specific primers for GAPDH (sense primer 5′-CCACCCATGGCAAATTCCATGGCA-3′; antisense primer 5′-TCTAGACGGCAGGTCAGGTCCACC-3′). PCR reactions were performed using GeneAmp RNA PCR kit (Perkin Elmer), and DNA products analysed on a 1.2% agarose/EtBr gel. A 600 bp fragment is amplified, as observed for both normal (N) and tumor (T) tissues from HNSCC patients (WSU62 and 63). Appropriate positive and negative controls are indicated

caspase precursors, *Bcl-W*, *Bax*, and *Bag-1*, but might survive apoptotic signals through the overexpression of apoptosis inhibiting molecules, including *Akt2* and *IAP* (inhibitor of apoptosis) (Ambrosini *et al*., 1997; Granville *et al*., 1998). Also shown in Figure 2B, cancer cells overexpressed signaling molecules participating in the MAP kinase pathway, including *ERK1*, all isoforms of *JNK* (1-3), two *p38* related MAPKs, *p38* and *ERK6*, and their upstream activators, *MEKK3* and *MKK6*, which is likely to contribute to the enhanced growth stimulation in these cells. Interestingly, the hyperplastic tissue exhibited a pattern of gene expression nearly identical to that of the adjacent normal epithelium, being only *cyclin D1* and DP_2 over-

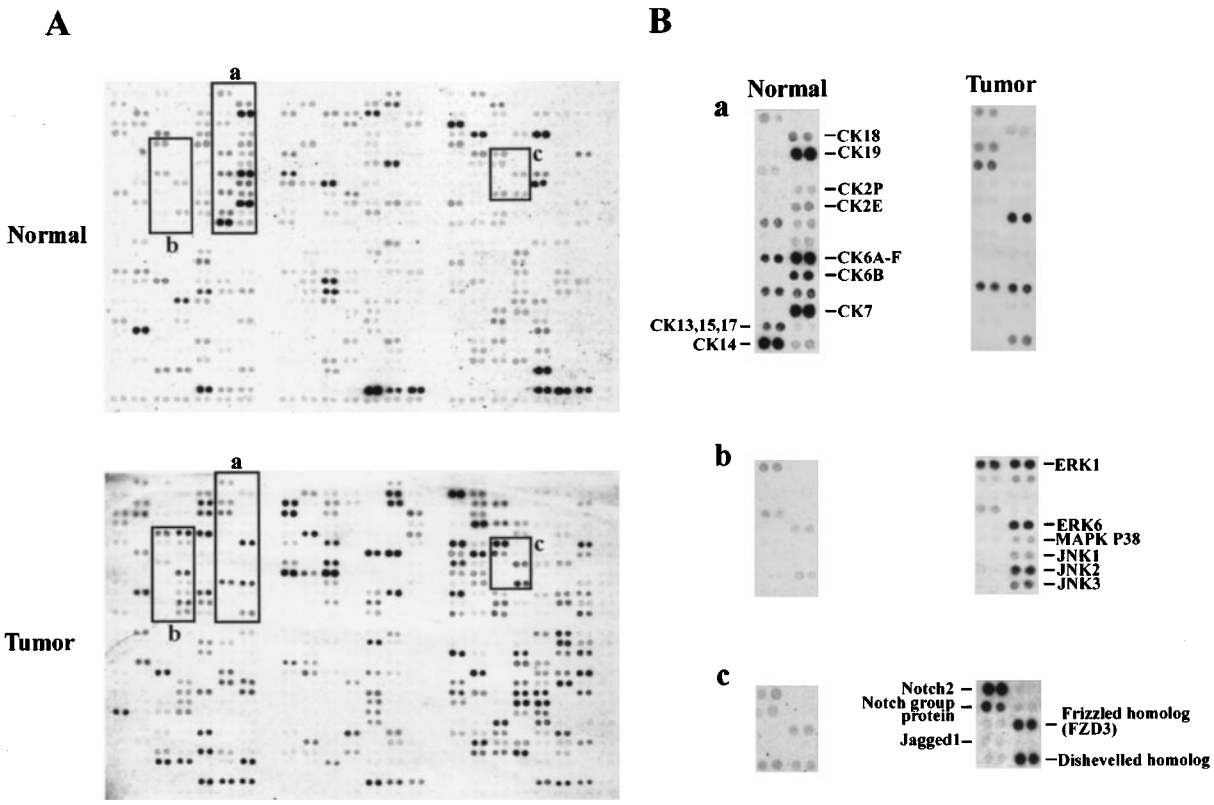

Figure 2 Analysis of gene expression in HNSCC using cDNA arrays. For each HNSCC tissue set, AcDNA probes were prepared and used simultaneously for the hybridization of nylon membranes arrayed in duplicate with human cancer and housekeeping genes. Probes were synthesized using 2 μl of cDNA for each reaction from tissue sets (normal and tumor), 2 μl of dNTP mix (2.5 mM of dGTP, dATP, dTTP and 0.5 mM of dCTP; Perkin Elmer), 75 μl ddH_2O, 10 μl 10$\times$ PCR reaction buffer and 4 μl 10 mM PCR primers (CLONTECH Smart kit), 5 μl 10 mCi/ml α-^{33}P dCTP and 2 μl Taq polymerase (Perkin Elmer). Each sample was mixed and used to synthesis complex cDNA probe by PCR (1 min at 95°C, then 30 cycles of 15 s at 95°C, 5 s at 65°C and 5 min at 68°C, and then cooled to 4°C). For each sample, 3–4 independent PCR reactions were carried out and combined after purification (PCR SELECT-II columns; 5Prime-3Prime, Inc.). The specific activity of each cDNA probe was assessed by scintillation counting. Human cancer cDNA expression arrays (CLONTECH) and the conditions used for hybridization were essentially as described in the manufacturer's protocol. After extensive washes, the membranes were analysed by PhosphorImaging (Molecular Dynamics) and auto-radiography. The hybridization of the cDNAs on the arrays for each of the samples (normal and tumor) was documented, and the identity of the genes determined from the relative position, as provided by the manufacturer. The comparative level of expression for each gene was assessed by PhosphoImaging, and expressed relative to that of the housekeeping genes (GAPDH). Pattern of gene expression for a representative tissue set from the same HNSCC patient is shown. Differentially expressed genes in three or more HNSCC tissue sets were considered of likely biological significance, and examples of those are indicated

expressed, which might correlate with increased cell proliferation. Thus, collectively these results demonstrate that squamous cells exhibit a distinct pattern of gene expression, which might help explain many of the cellular abnormalities described in this tumor type.

Unexpectedly, genes involved in the *wnt* and *notch* signaling pathway, were found to be highly represented in tumor tissues (2–5-fold). High expression levels of some of these genes have been demonstrated in many neoplasias and may have an implication in maintaining an undifferentiated epithelium (Liu *et al*., 1996; Shelly *et al*., 1999). For *wnt*, two *wnt* receptors, *frizzled* and *FDZ3*, and their downstream targets, *dishevelled* and *β-catenin* (Wodarz and Nusse, 1998) were highly expressed. In the case of *notch*, the detection of both receptor and ligand (*notch* and *jagged*, respectively) also suggests strongly their constitutive activation (Artavanis-Tsakonas *et al*., 1999). Furthermore, two of the *fringe* genes, *Manic* and *Lunatic*, which encode pioneer secretory proteins that modulate Notch-ligand interactions (Panin *et al*., 1997) were similarly highly represented. Thus, together these findings support an unexpected role for the *notch* signaling system in squamous cell carcinogenesis. Their precise role in the pathogenesis of HNSCC is currently unknown, and warrants further investigation.

Taken together, we can conclude that the use of LCM and cDNA arrays has allowed the detailed analysis of gene expression in HNSCC, and provided the first evidence for the feasibility of performing a comprehensive molecular characterization of normal, premalignant, and malignant HNSCC cells. Although data obtained involved a limited set of tissue samples, a general trend is already observed, implicating cell cycle regulating and signaling molecules, growth and angiogenic factors, matrix degrading proteases, and survival and apoptotic molecules. Furthermore, we obtained evidence implicating, for the first time, the *notch* and *wnt* pathways in squamous cell carcinogenesis. Further analysis of a more extensive sample collection using conventional and these recently available technologies will make it possible to define a pattern of gene expression in a tumor progression model of HNSCC. This experimental approach is also expected to facilitate the identification of candidate markers potentially correlated with malignancy, thus providing valuable tools of diagnostic and prognostic value to study premalignant lesions.

Table 2 Genes highly represented in HNSCC

Cell cycle		Signaling molecules	
L33264	cdc-2 Related kinase	X60811	ERK1
X59768	Cyclin D1	X79483	ERK6
U11791	Cyclin H	U82532	GDI Dissociation protein
U40345	p19INK4D	L35253	MAP kinase p38
U18422	DP-2	L26318	JNK1
		L31951	JNK2
Angiogenesis		U34819	JNK3
		U39657	MKK6
L12350	Thrombospondin2 precursor	U78876	MEKK3
U43142	VEGF-C	M31470	Ras-like protein (TC10)
X07819	MMP-7	L25080	RhoA
X07820	MMP-10		
D50477	MMP-16	Apoptosis	
Z30183	TIMP-3		
U76456		U45878	Inhibitor of Apoptosis Protein
		U37448	Caspase 7 precursor
Growth Factors and Oncogenes		U60520	Caspase 8 precursor
		U28014	Caspase 5 precursor
M11730	HER2	M77198	AKT2
M37722	bFGFR 1	U59747	BCL-W
Z12020	MDM2	U78798	TRAF2
M31213	c-RET	S83171	BAG-1
D17517	SKY	L22474	BAX
X87838	β-Catenin		
X14445	FGF 3	WNT/Notch signaling system	
M37825	FGF 5		
X63454	FGF 6	M73980	Notch 1
M65062	IGFBP5	U77493	Notch 2
M60718	HGF precursor	M99437	Notch group protein (N)
K03222	TGF-α	AF028593	Jagged 1
X02812	TGF-β	L37882	Frizzled
M96956	EGF Cripto protein CR1 and 2	U82169	Frizzled homolog (FZD3)
X06374	PDGF-a	U46461	Dishevelled homolog
X02811	c-SIS	U43148	Patched homolog
M74088	APC	U94352	Manic fringe
		U94354	Lunatic fringe

Gene expression pattern for each HNSCC tissue set (WSU 1, 51, 58, 62, 63) was analysed by PhosphorImaging and the relative amount of expression was compared with those of housekeeping genes. Those genes judged to be differentially expressed at >twofold in at least three of the cancer tissue sets were considered of likely biological significance, and are listed in their corresponding functional groups. The GeneBank access number for each gene is included

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