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Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma

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Genomes of solid tumors are characterized by gains and losses of regions, which may contribute to tumorigenesis by altering gene expression. Often the aberrations are extensive, encompassing whole chromosome arms, which makes identification of candidate genes in these regions difficult. Here, we focused on narrow regions of gene amplification to facilitate identification of genetic pathways important in oral squamous cell carcinoma (SCC) development. We used array comparative genomic hybridization (array CGH) to define minimum common amplified regions and then used expression analysis to identify candidate driver genes in amplicons that spanned <3 Mb. We found genes involved in integrin signaling (*TLN1*), survival (*YAP1*, *BIRC2*), and adhesion and migration (*TLN1*, *LAMA3*, *MMP7*), as well as members of the hedgehog (*GLI2*) and notch (*JAG1*, *RBPSUH*, *FJX1*) pathways to be amplified and overexpressed. Deregulation of these and other members of the hedgehog and notch pathways (*HHIP*, *SMO*, *DLL1*, *NOTCH4*) implicates deregulation of developmental and differentiation pathways, cell fate misspecification, in oral SCC development.

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Introduction

Oral squamous cell carcinoma (SCC) remains a significant health problem (Parkin *et al*., 1999). The 5-year survival rate, at 40%, is among the worst of all sites in the body and has not improved over the past 40 years (Parkin *et al*., 1999). Oral epithelial dysplasia often precedes SCC development. Hence, there is both a need for more effective therapies and also the opportunity to recognize premalignant lesions and initiate chemoprevention, ideally targeting specific genes deregulated in oral cancer. Oral SCC development and progression from dysplasia is associated with accumulation of genetic and epigenetic alterations. In particular, deletions of 3p and 9p and activation of telomerase are thought to be among the earlier events, and are followed by amplification of *CCND1* and deletions involving 8p, 17p, 13q and 18q. Upregulation of *EGFR* and inactivation of p53 are also observed frequently (Kim and Califano, 2004). Specific therapies targeting some of these genes are currently being evaluated in the clinic (Mao *et al*., 2004). Nevertheless, other candidate oncogenes and tumor suppressors could be identified by analysis of genes mapping to regions of genomic aberration in oral SCC, but this approach is not straightforward, since these regions often encompass whole chromosome arms, making identification of key genes difficult and requiring extensive evaluation of many genes. Generally, investigations have focused on identification of single driver genes within a region of aberration. For example, *CCNL1* and *LRP2* were identified in this way as genes overexpressed in oral SCC (Redon *et al*., 2002; Garnis *et al*., 2004).

Although there is some controversy surrounding the functional significance of the many DNA copy number changes seen in tumor genomes, amplifications, defined as regions of focal high-level copy number change (Snijders *et al*., 2003), are likely to represent alterations continuously under selection for tumor growth, since studies indicate that the amplified DNA is unstable (Miele *et al*., 1989; Murnane and Sabatier, 2004; Roth and Andersson, 2004; Shimizu *et al*., 2005). On the other hand, there are many other ways to upregulate gene expression in addition to increasing the copy number of the gene (Albertson *et al*., 2003). Bearing in mind these observations, we hypothesized that even rarely occurring amplicons would be informative regarding genes

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important in tumor development, the rationale being that expression of a gene might be altered by copy number in some cases, but more often its expression could be altered by other mechanisms. Furthermore, the pathway in which the gene functions might be altered at a number of points by disrupting upstream or downstream genes by different mechanisms. Therefore, genes mapping in amplicons might help to focus attention on pathways likely to be deregulated in tumors and amplicons, even rare ones, encompassing a limited region of the genome can facilitate identification of candidate pathways because the number of genes in the region is likely to be smaller. This approach takes advantage of the selection by the tumor for amplification of the region to identify candidate pathways that may be deregulated in cancer, rather than focusing on definitive identification of the driver genes for particular amplicons. To apply this logic to oral SCC, we used array CGH to obtain genome-wide information on copy number alterations. We identified regions of recurrent gene amplification and carried out expression analysis of genes mapping within these amplified regions. These analyses found genes involved in integrin signaling, cell survival, and adhesion and migration to be amplified and/or overexpressed. They also identified members of the hedgehog and notch pathways to be amplified and/or overexpressed. Further analyses revealed deregulated expression of other members of these pathways, which control normal proliferation and differentiation (Ingham and McMahon, 2001; Hansson *et al*., 2004). These observations implicate misspecification of cell fate in the development of oral cancer and suggest the possibility of applying new therapeutic approaches targeting members of the hedgehog and notch signaling pathways (Pasca di Magliano and Hebrok, 2003; Watkins and Peacock, 2004).

Results and discussion

We carried out a genome-wide analysis of copy number aberrations in 89 oral SCC (Figure 1a) taken from four different sites in the oral cavity (Table 1). This analysis revealed a number of frequent low-level gains and losses (Figure 1b) and 18 regions of recurrent amplification (Table 2), a number of which contained genes found previously to be amplified and/or overexpressed in oral SCC. Hierarchical clustering of the array CGH data of all 89 tumors revealed two main branches in the dendrogram (Figure 2a), one of which is significantly enriched with tumors with mutations in *TP53* exons 5–8 (Fisher exact test *P*-value = 0.001). Regardless of position in the dendrogram, low level copy number alterations significantly associated with *TP53* mutational status after maxT (Westfall and Young, 1993) correction for multiple testing included −8p, +distal 8q, −10q, −11q and −18q (Figure 2b). In addition, we observed that mutation of *TP53* was positively correlated with amplification of *CCND1* (Fisher exact test *P*-value = 0.009), confirming a previous report (Mineta *et al*., 1997) and with amplification of *EGFR* (Fisher exact test *P*-value = 0.036).

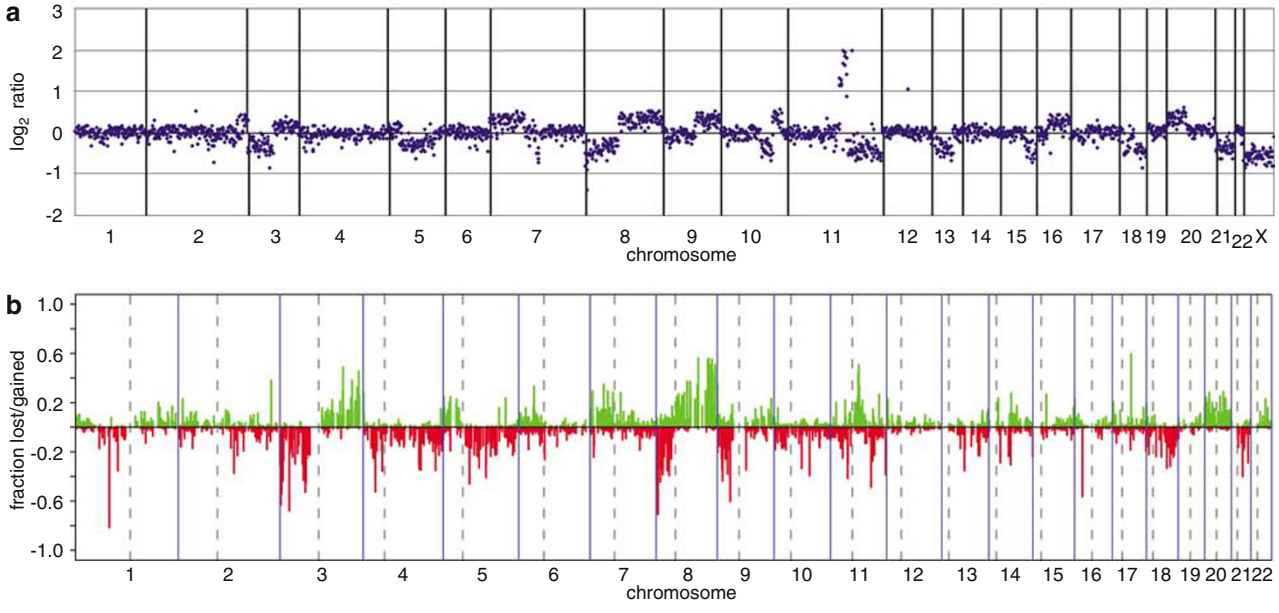


Figure 1 Genome-wide analysis of copy number aberrations in 89 oral SCC. (**a**) Normalized copy number ratios of genomic DNA from an oral SCC from the buccal mucosa of a male patient compared to normal female reference DNA. Plotted is the normalized $\log_2$ratio for each clone sorted by chromosome and ordered according to genome position from the p-arm to the q-arm. Multiple low-level DNA copy number aberrations are present, including loss of chromosome 3p, 5q, 8p, 18 and 21 and gain of chromosome 7p, 8q, 9q and 20p. This tumor also has an amplification at 11q13 including *CCND1*. (**b**) Frequency of gains, indicated by the green bars ranging from 0 to 1, and losses, indicated by the red bars ranging from 0 to −1, in 89 oral SCC for each clone. The most recurrent regions of DNA copy number loss are on chromosomes 3p, 4, 5q, 8p, 9p, 18 and 21, while recurrent regions of copy number gains are on chromosomes 3q, 8q, 11q and 20 in general agreement with previous studies of oral SCC. (Wolff *et al*., 1998; Huang *et al*., 2002)

Table 1 Patient characteristics

Specimen	*Differentiation*	*Location*	*Sex*	*Age*	TP53 *mutation*
3103	Moderate	Buccal mucosa	M	80	Exon 8, codon 280 AGA to ACA (arg to thr)
3144	Poor	Buccal mucosa	F	68	Exon 6, codon 216 GTG to ATG (val to met)
3482	Moderate	Buccal mucosa	M	46	Not done
3525	Moderate	Buccal mucosa	F	66	Exon 8, codon 282 CGG to TGG (arg to trp)
3887	Well	Buccal mucosa	M	66	No mutation
4833	Moderate to poor	Buccal mucosa	F	67	Exon 8, codon 273 CGT to CAT (arg to his)
4837	Moderate to poor	Buccal mucosa	F	56	No mutation
5403	Well	Buccal mucosa	F	80	No mutation
5731	Moderate	Buccal mucosa	F	79	No mutation
5782	Moderate to poor	Buccal mucosa	F	81	No mutation
5864	Moderate	Buccal mucosa	M	35	No mutation
6166	Well	Buccal mucosa	F	76	No mutation
6352	Well	Buccal mucosa	M	48	No mutation
6420	Moderate	Buccal mucosa	M	40	Exon 6 codon 196 CGA to TGA (arg to STOP)
6440	Poor	Buccal mucosa	F	52	No mutation
6477	Well	Buccal mucosa	F	90	No mutation
6856	Well	Buccal mucosa	M	64	No mutation
2861	Moderate	Floor of mouth	M	69	No mutation
2868	Moderate to well	Floor of mouth	F	62	No mutation
2962	Moderate	Floor of mouth	M	64	No mutation
3333	Moderate	Floor of mouth	F	56	No mutation
3501	Moderate	Floor of mouth	F	74	No mutation
3816	Moderate	Floor of mouth	M	69	No mutation
4836	Moderate to poor	Floor of mouth	M	60	No mutation
4961	Moderate	Floor of mouth	M	74	No mutation
5645	Poor	Floor of mouth	M	49	Exon 6, codon 220 TAT to TGT (tyr to cys)
5699	Well	Floor of mouth	F	68	No mutation
5730	Moderate	Floor of mouth	F	83	Exon 5 base -1 (last base INTRON 4-5 G to T)
5771	Well	Floor of mouth	F	63	No mutation
5954	Moderate	Floor of mouth	M	82	No mutation
6063	Moderate	Floor of mouth	F	91	No mutation
6362	Moderate	Floor of mouth	M	82	Exon 5 codon 132 AAG to GAG (lys to glu)
6520	Moderate	Floor of mouth	M	56	Exon 6 codon 213 CGA to CGG (arg to arg) silent
6879	Moderate	Floor of mouth	M	62	Exon 6 codon 213 CGA to CGG (arg to arg) silent
2565	Moderate to well	Gingiva	F	75	Not done
3444	Well	Gingiva	F	88	No mutation
3558	Well	Gingiva	M	75	No mutation
3883	Well	Gingiva	F	84	Exon 8 codon 282 CGG to TGG (arg to trp)
4118	Well	Gingiva	F	80	No mutation
4142	Moderate	Gingiva	M	70	No mutation
4397	Well	Gingiva	M	86	Exon 7 codon 248 CGG to CAG (arg to glu)
4456	Well	Gingiva	F	66	No mutation
4895	Well	Gingiva	M	68	Not done
5790	Moderate	Gingiva	F	73	No mutation
5793	Well	Gingiva	F	63	Exon 8 codon 274 GTT to CTT (val to leu)
5833	Moderate to well	Gingiva	M	35	No mutation
5916	Well	Gingiva	M	88	No mutation
6200	Well	Gingiva	F	77	No mutation
6253	Well	Gingiva	F	78	No mutation
6376	Moderate	Gingiva	M	36	No mutation
6378	Well	Gingiva	M	39	No mutation
6401	Moderate	Gingiva	F	83	No mutation
6672	Well	Gingiva	M	55	Exon 5 codon 176 TGC to TTC (cys to phe)
6765	Moderate	Gingiva	M	64	No mutation
6831	Well	Gingiva	M	84	No mutation
280	Well	Tongue	F	26	No mutation
1067	Well	Tongue	F	30	Exon 6 codon 196 CGA to TGA (arg to STOP)
1424	Well	Tongue	M	27	No mutation
2042	Moderate	Tongue	F	29	Exon 6 codon 196 CGA to TGA (arg to STOP)
3486	Well	Tongue	M	73	Not done
3666	Well	Tongue	M	62	No mutation
3720	Well	Tongue	F	69	Not done
3800	Moderate	Tongue	M	75	Not done
3927	Moderate	Tongue	F	77	No mutation
4052	Moderate to well	Tongue	F	26	No mutation
4756	Moderate	Tongue	F	31	No mutation
5463	Well	Tongue	M	50	No mutation
5814	Poor	Tongue	M	64	No mutation
5832	Moderate to well	Tongue	F	55	No mutation
5877	Moderate	Tongue	M	77	No mutation

Table 1 (*continued*)

Specimen	*Differentiation*	*Location*	*Sex*	*Age*	TP53 *mutation*
5885	Well	Tongue	F	48	No mutation
5911	Well	Tongue	M	54	No mutation
5918	Well	Tongue	F	40	No mutation
5941	Moderate	Tongue	M	56	No mutation
6035	Poor	Tongue	F	55	No mutation
6198	Well	Tongue	F	47	No mutation
6422	Well	Tongue	F	42	No mutation
6508	Moderate	Tongue	F	48	No mutation
6659	Well	Tongue	M	66	No mutation
6666	Moderate	Tongue	F	62	No mutation
6675	Well	Tongue	F	53	No mutation
6732	Well	Tongue	F	38	Not done
6987	Well	Tongue	M	52	Not done
6799	Moderate to well	Tongue	M	68	Not done
6807	Moderate	Tongue	F	41	Not done
6818	Well	Tongue	F	78	Not done
6929	Moderate	Tongue	F	53	Not done
6988	Moderate	Tongue	M	60	Not done
7107	Moderate	Tongue	M	36	Not done

We then focused on the nine amplicons with boundaries spanning less than 3 Mb (Table 2, Figure 3). We identified two regions containing genes frequently amplified and/or over-expressed in oral SCC, including *EGFR* ($n=10$ cases) at 7p11.2, and/or two separate, but co-amplified regions at 11q13 encompassing *CCND1*, *FGFR3*, *FGFR4* and *EMS1* in a 1.5 Mb amplicon and *PAK1* in a separate 0.9 Mb amplicon ($n=10$ cases). The remaining seven amplicons did not contain well-established oncogenes in oral SCC. Therefore, to identify the driver genes in these amplicons, we evaluated transcript levels of candidate genes mapping in the minimum amplicon in a set of 24 tumors using the quantitative reverse transcription polymerase chain reaction (RT–PCR) and compared the expression levels to normal samples from the tongue ($n=2$) and buccal mucosa ($n=1$). These analyses (Figure 4, Supplementary Table A and Supplementary Figure A) indicate greater than twofold upregulation of expression compared to the normal tissue of *GLI2* at 2q14.2 (11/23 tumors for which an expression measurement was available), *RBPSUH* at 4p15.2 (4/14 tumors), *TLN1* at 9p13.3 (4/23 tumors), *FJX1* at 11p13 (15/17 tumors), *YAP1*, *BIRC2* and *MMP7* at 11q22 (4/23, 4/23, 17/28 tumors, respectively), *LAMA3* at 18q11.2 (19/23 tumors) and *JAG1* at 20p12.2 (10/23 tumors). In all cases, we observed increased expression of these genes when they were amplified, but more frequently they were overexpressed without amplification, suggesting that they are likely candidate driver genes for amplification of their respective amplicons. We consider *BIRC3* and *KIAA0746* less likely to be driver genes, since we observed little upregulation of these genes when amplified. Furthermore, they are expressed in lymphatic tissue and thus, overexpression in tumor samples probably reflects inflammatory infiltrate (Supplementary Table A). On the other hand, we observed high levels of expression of *FJX1* in lymph nodes, but not in a buccal mucosa sample with lymphocytic infiltrate. Since *FJX1* was highly upregulated when amplified, we retained *FJX1* as a candidate driver gene for amplification at 11p13.

Based on the known functions of the candidate genes, we can propose plausible roles in tumorigenesis, including deregulation of transduction of integrin signaling (*TLN1*) (Nayal *et al*., 2004), opposition to apoptosis (*YAP1* and *BIRC2*), and adhesion and migration (*TLN1*, *LAMA3*, *MMP7*) (Lohi, 2001; Nayal *et al*., 2004). However, we were particularly interested in the observed amplification and overexpression of *GLI2*, *JAG1*, *RBPSUH* and *FJX1*, members of the hedgehog and notch pathways. These ontogenetic networks function in cell fate specification and are widely conserved across phyla. Deregulated hedgehog signaling is a hallmark of basal cell carcinomas of the skin (Ruiz i Altaba *et al*., 2002) and odontogenic keratocysts in the oral cavity (Ohki *et al*., 2004), both of which are phenotypically distinct from SCC. On the other hand, notch signaling promotes differentiation in skin (Lefort and Dotto, 2004) and loss of function of notch is permissive for tumor formation in mouse models (Nicolas *et al*., 2003). Although two expression array studies found upregulation of some members of the wnt and notch signaling pathways in head and neck SCC (Leethanakul *et al*., 2000; Ha *et al*., 2003), the hedgehog and notch pathways have not been previously characterized in oral squamous cell tumorigenesis. Therefore, we investigated whether expression levels of other members of the hedgehog and notch pathways were altered in oral SCC.

Hedgehog signaling involves secretion of the hedgehog morphogens, Sonic (*SHH*), Indian (*IHH*) and Desert (*DHH*) hedgehog, which interact with Patched (*PTCH*) to relieve inhibition of Smoothened (*SMO*), resulting in activation of the transcription factors, *GLI1*, *GLI2* and *GLI3* (Ingham and McMahon, 2001; Taipale and Beachy, 2001). We observed that *GLI2* expression was positively correlated with expression of *GLI1*

Table 2 Recurrent amplicons in 89 oral SCC

Tumors	*Chrom.*	*Amplicon copy number range (log_2 ratio)*	*Proximal flanking clone*	*STS*	*Start (bp)*	*Distal flanking clone*	*STS*	*End (bp)*	*Size (Mb)*	*Selected genes in amplicons*
4756, 6929	2q14.2	0.5–1.5	RP11-438O12		121,225,342	RP11-416H1	D2S343	122,458,621	1.2	GLI2
3103, 3482	3q24-25	1.0–1.8	RP11-72E23	D3S1557	146,527,612	RP11-65L11	AFM277WF9	152,627,100	6.1	TM4SF1
3883, 4052, 4961	4p15.2	0.7–2.9	RP11-118C24	SHGC-24618	25,331,058	RP11-194B9	SHGC4-273	28,103,518	2.8	*RBPSUH*; *STIM2*
5918, 6362	5p13.2	0.7–0.9	RP11-67P13	AFMA297WA5	32,233,715	RP11-9G14	D5S634	41,329,416	9.1	*RAD1*; *SKP2*; *IL7R*
1424, 5730	6q12	1.0	RP11-277K21	AFMB291ZB5	64,091,403	RP11-2M9	AFM295TB3	71,712,187	7.6	*PTP4A1*; *EGFL11*
1067, 2042, 3816, 4397,	7p11.2	0.7–2.6	GS1-6E1		53,465,093	RP11-251I15	D7S499	55,349,162	1.9	EGFR
5463, 5699, 5793, 5832,	7q21.2	2.3–2.6	CTB-141D22		90,029,465	CTD-2007G21	U31384	93,098,318	3.1	*FZD1*, *CDK6*
5877, 6799	8p12	1.1–1.2	RP11-210F15	SHGC-20486	36,452,678	RP11-262I23	SHGC-12674	39,744,917	3.3	*BAG4*; *FGFR1*; *TACC1*; *ADAM9*
6988, 3800, 5790										
3144, 5918										
3482, 3501, 5864, SCC094	9p24.1	0.9–1.4	RP11-12N24	SHGC-34067	5,223,942	RP11-50C21	SHGC-18065	10,431,717	5.2	*UHRF2*; *JMJD2C*; *PTPRD*
1067, 6362, 6818	9p13.3	0.6–2.1	RP11-37F22	SHGC-35868	34,971,713	RP11-39I6	SHGC-32868	36,174,984	1.2	TLN1
280, 4833, 6420, 6362, 6831	11p13	0.6–1.1	RP11-90F13	AFMA081TG5	34,957,558	RP11-187A8	SHGC-6028	36,559,560	1.6	*CD44*; *FJX1*, *TRAF6*
280, 2042, 2962	11p11.2	0.6–1.0	RP11-102E22	SHGC-13806	44,278,905	CTD-2244P3		47,245,301	3.0	*MAPK8IP1*; *BHC80*
2042, 4833, 5730, 5771,	11q13.3	0.7–3.6	CTD-2080I19	RH7839	68,482,959	RP11-120P20	SHGC-4518	70,129,383	1.6	*CCND1*; *FGF3*,
4397, 5790, 6508, 6672,										*4*, *19*; *EMS1*
6799, 6988										
6672, 6988	11q13.5	1.4–1.8	CTC-352E23	RH52308	76,097,938	RP11-98G24	SHGC-31540	77,013,406	0.9	PAK1
1067, 2861, 3800, 5833,	11q22	0.7–2.6	RP11-5G24	SHGC-10856	101,181,950	RP11-817J15	SHGC-11011	101,955,349	0.8	*YAP1*; *BIRC2*;
6420										*BIRC3*; *MMP7*
3800, 4961, 5463, 5814,	12q15	0.4–1.3	RP11-5J6	SHGC-3797	66,882,015	RP11-92P22	SHGC-35465	74,052,886	7.2	*MDM2*; *PTPRR*
6362										
2861, 2868, 6508, 6988	18q11.2	0.4–1.2	CTD-2100E13	STSG21909	19,275,988	RP11-59E12	AFM164ZC1	19,809,522	0.5	LAMA3
1067, 3482, 4961	20p12.2	0.5–1.1	RMC20P160	WI-7829	10,282,059	RMC20P178	D20S186	11,518,795	1.2	JAG1

($R^2 = 0.7$) and *PTCH* ($R^2 = 0.5$), consistent with observations in other systems in which it has been shown that these genes are direct targets of *GLI2* (Ingham and McMahon, 2001; Regl *et al*., 2002; Agren *et al*., 2004). We observed that *SMO* was more than twofold downregulated in the majority of tumors (11/23 for which an expression measurement was available). Limited analysis of oral SCC cell lines has shown that *SHH* is expressed in several and further that the pathway is likely to be active in at least one cell line, since growth can be inhibited by exposure to the Smoothened inhibitor cyclopamine (Nishimaki *et al*., 2004). However, we found no expression of *SHH*, *IHH* or *DHH* in normal tissue and only rarely detected expression in tumors (0/23, 0/21 and 1/5 tumors for which an expression measurement was available, respectively), whereas we did observe expression of *SHH* in the colon carcinoma cell line HCT116 in agreement with a previously published report (Berman *et al*., 2003), indicating that the assay is capable of detecting *SHH* expression. On the other hand, although we detected expression of *HHIP*, a negative regulator of hedgehog signaling in normal tissue, we found no expression in the majority of tumors (16/20 for which an expression measurement was available). Taken together, these observations suggest that endogenous ligand activation of hedgehog signaling is not inducing *GLI2* upregulation in oral SCC, in contrast to small-cell lung carcinoma (Watkins *et al*., 2003) and tumors from tissues of endodermal origin, including the esophagus, stomach and pancreas (Berman *et al*., 2003; Watkins and Peacock, 2004). While the mechanism for *GLI2* upregulation in oral SCC is not known, possible routes include activation of *GLI2* via other pathways, such as fibroblast growth factor (Ruiz i Altaba *et al*., 2002) or the observed downregulation of *HHIP*, a negative regulator of hedgehog signaling (Chuang and McMahon, 1999; Chuang *et al*., 2003). Notch signaling is initiated by interaction of ligands (e.g. DLL1, JAG1) with membrane-bound notch resulting in cleavage of the notch protein. The released notch intracellular domain enters the nucleus and interacts with the transcription factor, RBPSUH, leading to expression of 'hairy enhancer of split' genes (Lefort and Dotto, 2004; Weng and Aster, 2004). Although notch signaling promotes differentiation in skin (Lefort and Dotto, 2004), in oral SCC, we found amplification and overexpression of *JAG1* and *RBPSUH*, two genes central to notch signaling. In addition, we observed amplification of *FJX1*, the human homologue of *four-jointed*, which acts in *Drosophila* as a second signal downstream of notch in leg development (Buckles *et al*., 2001) and in regulation of polarity in ommatidial development (Zeidler *et al*., 1999). The function of *FJX1* in humans is currently unknown, but amplification of this gene together with *JAG1* and *RBPSUH* in oral SCC suggests that deregulation of notch signaling is likely contributing to development of these tumors. Therefore, we examined the expression of other notch pathway genes in tumors and found that expression of *HES1* increased with increasing expression of *JAG1* and *DLL1* (Supplementary Table A), suggesting that notch signaling was active in the tumors. We observed that *DLL1* and *NOTCH4* were upregulated twofold relative to normal tissue (8/21 and 8/19 tumors, respectively), whereas we found a tendency for *NOTCH1*, *2* and *3* and *HES1* to be expressed at lower levels. Upregulation of *NOTCH4* is associated with breast and mammary cancer (Weng and Aster, 2004). Independent confirmation of our observations is provided by recent expression array studies, which found significant upregulation of *JAG1* and *NOTCH4* in oral SCC compared to normal tissue (Leethanakul *et al*., 2000; Ha *et al*., 2003). These studies together with the data reported here indicate a role for deregulation of notch signaling in oral cancer.

Skin and oral epithelia are continually renewed and show a developmental gradient from the basal layer to the outermost layer where cells are shed. In the oral cavity, epithelial stem cells located in the deep rete ridges divide rarely to give rise to transit amplifying cells, which are committed to differentiation, but divide a limited number of times prior to withdrawing from the cell cycle (Hume and Potten, 1979). Oral SCC progenitor cells are likely to be stem cells that have acquired appropriate mutations to allow them to proliferate abnormally and/or to render transit amplifying cells resistant to differentiation. Amplification and/or overexpression of members of the hedgehog and notch pathways in tumors suggest that deregulation of these pathways plays a role in misspecification of oral epithelial cell fates leading to tumor development. Since a number of the involved genes may be expressed in various forms with different growth promoting or repressing functions (Ascano *et al*., 2003; Aho, 2004) and the outcome of notch signaling is context dependent (Weng and Aster, 2004), identification of the precise roles of these pathways in oral cancer awaits further characterization of the overexpressed proteins and their pathways.

Materials and methods

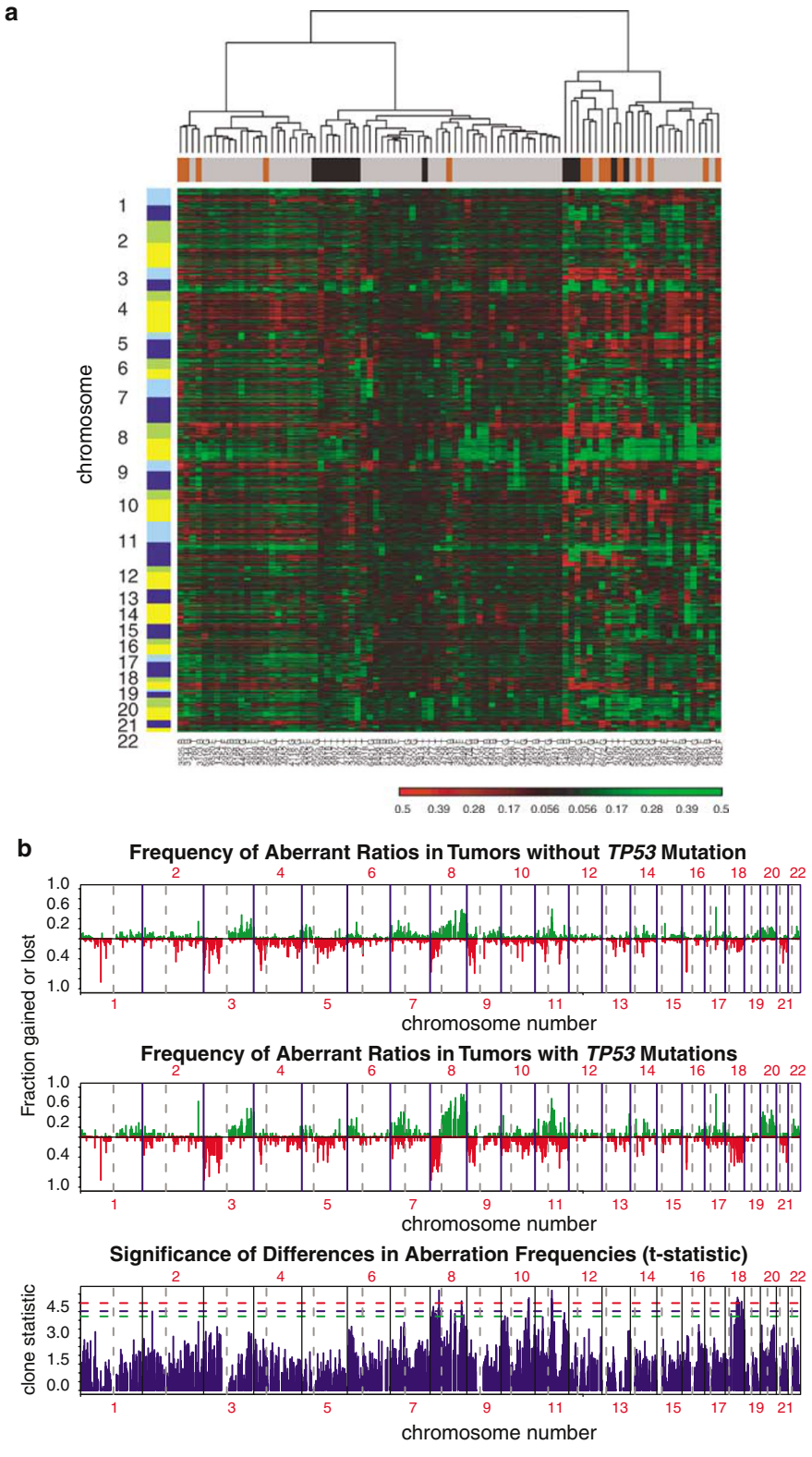
Tumor samples

Oral SCC and normal tissue specimens and associated clinical data were obtained through the UCSF Oral Cancer Tissue Bank. Normal cervical lymph nodes were removed as part of the surgical treatment for benign oral disease and were also obtained through the UCSF Oral Cancer Tissue Bank. All tissues were fixed in formalin prior to processing in paraffin. Patient consent was obtained for use of all specimens. Prior to nucleic acid extraction, we stained the first and last sections with hematoxylin and eosin. We examined these sections to confirm the diagnosis of SCC prior to collection of material for nucleic acid extraction and to estimate the normal cell content of the regions selected for dissection, which varied from 60 to 90% epithelial cells. Patient samples and characteristics are provided in Table 1.

Isolation of DNA

We dissected tumor-rich regions from 15 consecutive 10 μm formalin-fixed paraffin-embedded tissue sections from routine

surgical biopsies and tumor resections. We de-paraffinized the sections by using three 10 min incubations with 1 ml xylene on a rocking table. We removed the xylene following centrifugation of the sections and then incubated the sections in 1 ml of absolute ethanol for 15 min at room temperature. After removal of the ethanol, we air-dried the sections and then incubated them overnight in 500 μl digestion buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, 0.5% SDS in H_2O). We



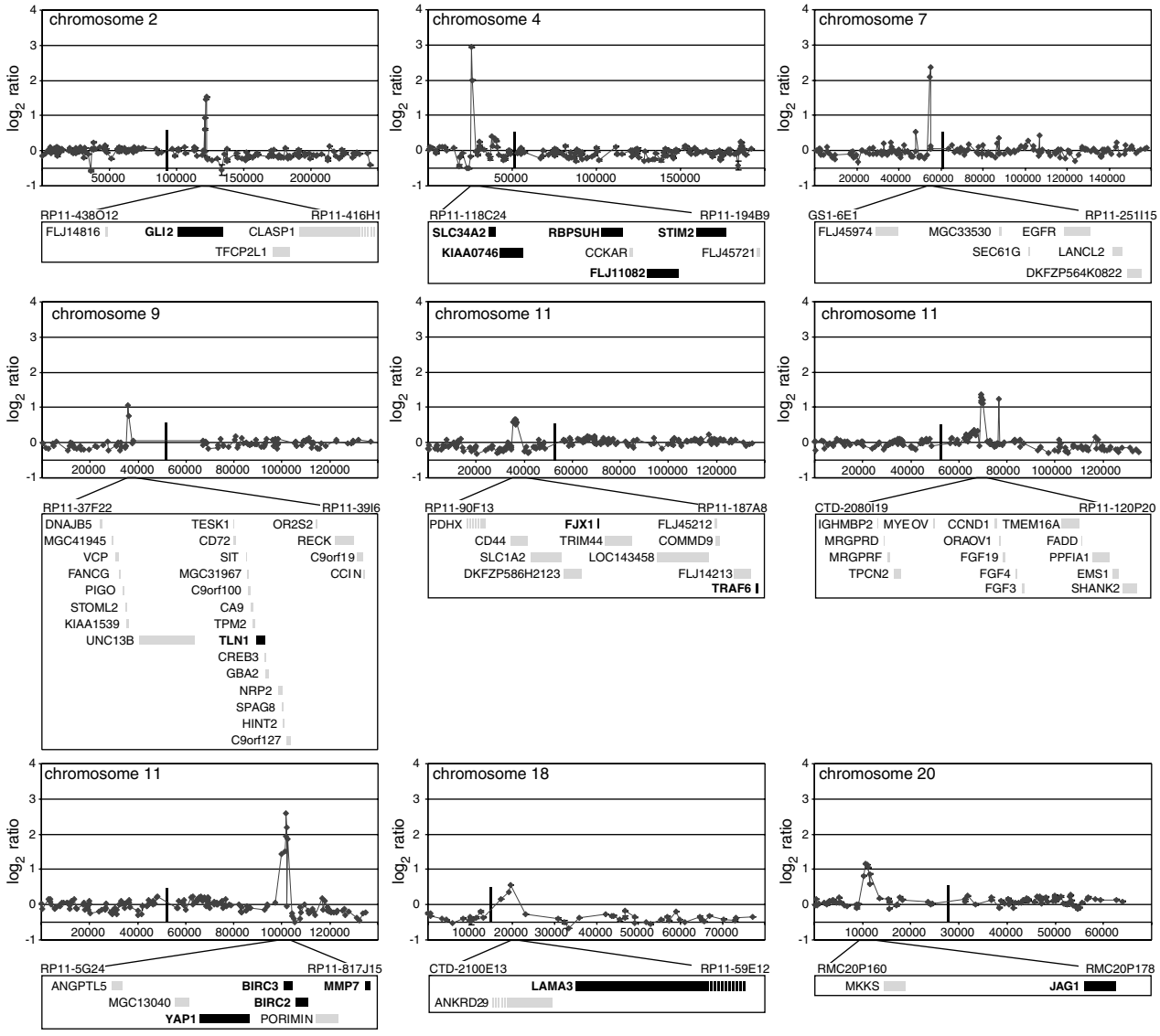


Figure 3 Overview of recurring DNA amplifications in 89 oral SCC determined by genome-wide array CGH. We compared the extent of amplicons in a series of tumors to determine the minimum recurrent region of amplification to facilitate identification of potential driver genes underlying each aberration. We show typical copy number profiles for nine recurrent regions of amplification that did not exceed 3 Mb (range 0.5–2.8 Mb) observed on chromosomes 2, 4, 7, 9, 11 (3 regions) and 18. For all chromosomes, we plotted the data according to position on the chromosome in kilobases using the July 2003 freeze of the draft human genome sequence (http://www.genome.ucsc.edu/) and indicate the position of the centromere by a vertical bar. The expanded view of the minimum common region of amplification, shown underneath each amplicon, includes the most proximal and distal flanking clones and the genes present in the region (UCSC July 2003 freeze). Since driver genes are likely to be overexpressed at the transcript level, we performed quantitative RT–PCR analysis of candidate driver genes (shown in black with bold lettering) in seven of the nine recurrent regions of amplification in a subset of tumors including amplifying tumors

Figure 2 Genomic analysis of 89 oral SCC. (**a**) Hierarchical clustering of 89 oral SCC based on their genome-wide DNA copy number profile. We represented individual clones as rows and ordered them by chromosome and genome position according to the July 2003 freeze of the human genome. Clones on the p-arm are indicated either in light blue or green, and clones on the q-arm in dark blue or yellow. We show acrocentric chromosomes in yellow or dark blue. Columns represent individual tumor samples. The *TP53* mutation status of tumors is indicated with a maroon box for *TP53* mutant tumors, a gray box for tumors with no detected mutation and a black box if the *TP53* status is unknown. Mutations in exons 5–8 of *TP53* (five buccal mucosa, three floor of mouth, four gingiva and two tongue) were found in 18% of the oral SCC. (**b**) Frequency plots of oral SCC with and without mutation in *TP53* exons 5–8. The top panels show the frequency with which we found aberrant ratios on individual clones in at least 10% of samples. For each clone, we show gains (indicated in green, ranging from 0 to 1) and losses (indicated in red, ranging from 0 to 1) using tumor-specific thresholds for 89 oral SCC sorted according to *TP53* mutation status. The bottom panel shows the level of significance of the difference (*t*-statistic) between the two sets of tumors at each clone. The dashed horizontal lines indicate $P = 0.01$ (green), $P = 0.05$ (blue) and $P = 0.001$ (red)

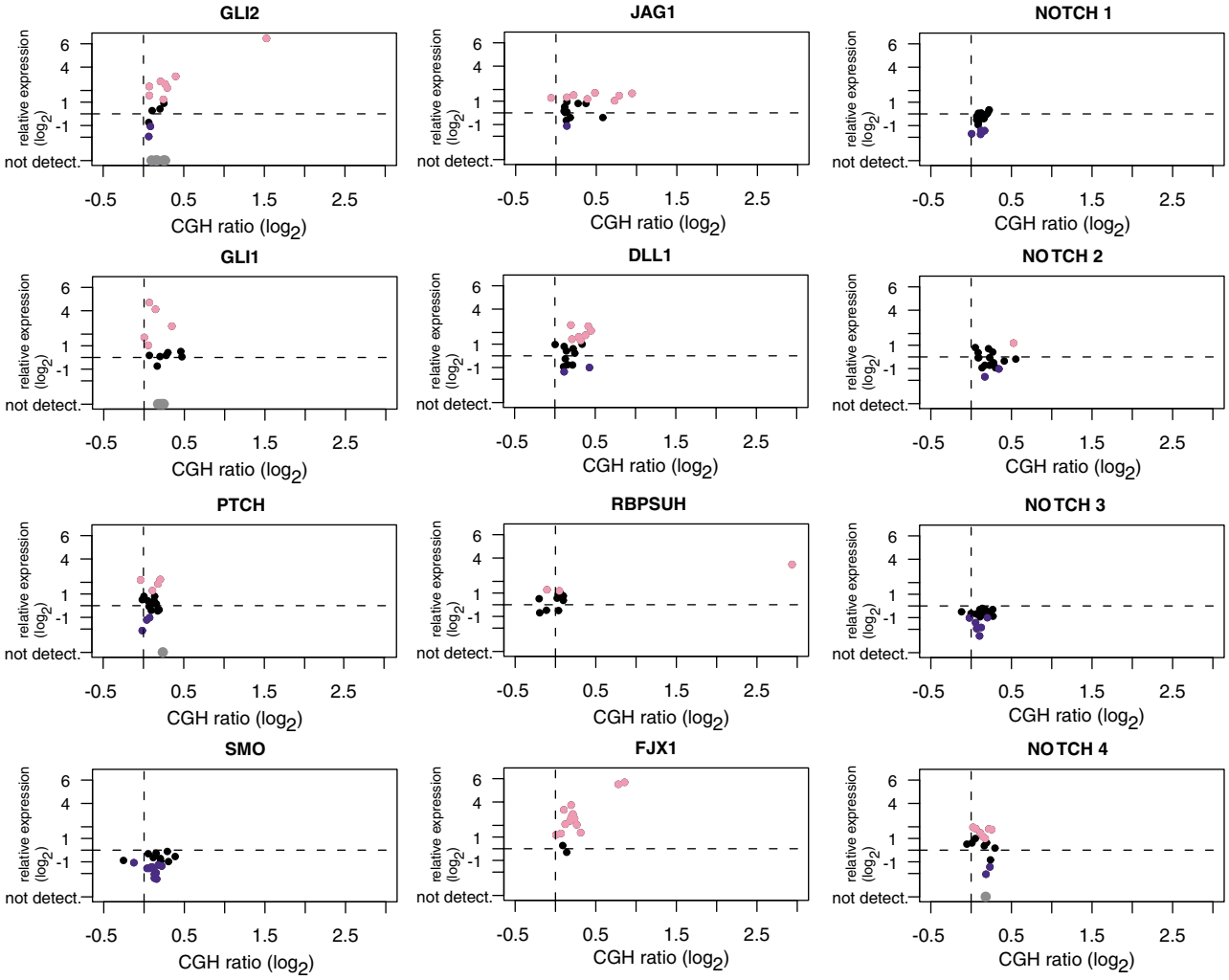


Figure 4 Expression of selected amplified genes and associated pathway members compared to the CGH $\log_2$ratio of the locus in oral SCC. For each gene we show expression levels in a series of tumors relative to the average expression in several normal oral tissue samples (Supplementary Table A) versus $\log_2$ratio observed on a BAC clone either containing or closest to each corresponding gene. We highlighted samples, which were more than twofold overexpressed relative to normal tissue in pink, samples downregulated more than twofold relative to normal tissue in light blue and samples in which expression of the gene was not detected in gray at the bottom of the plot. We observed that amplified genes (e.g. *GLI2*, *JAG1*, *FJX1*, *RBPSUH*) were always overexpressed when amplified, but more frequently genes were overexpressed without amplification (e.g. *GLI2*, *GLI1*, *JAG1*, *FJX1*, *RBPSUH*, *DLL1*, *NOTCH4*)

treated the sections with 400–500 μg of Proteinase K each day for three consecutive days at 55°C and then extracted DNA using phenol–chloroform–isoamyl alcohol (25 : 24 : 1). We precipitated the DNA with ethanol and ammonium acetate in the presence of 100 μg glycogen and collected the pellet by centrifugation. We dissolved the air-dried pellet in 15 μl H_2O and determined the DNA concentration by fluorometry.

Array CGH

We carried out array CGH as described previously (Snijders *et al*., 2003). Briefly, to label genomic DNA (600 ng), we used random priming to incorporate Cy3- or Cy5 dCTP in a 50 μl reaction. We hybridized labeled test (600 ng) and reference DNAs (300 ng) together with 100 μg human Cot-1 DNA for $\sim$48 h at 37°C to arrays of 2464 BAC clones each printed in triplicate (HumArray2.0, UCSF Comprehensive Cancer Center Microarray Core) (Snijders *et al*., 2001). We acquired 16-bit 1024 $\times$ 1024 pixel DAPI, Cy3 and Cy5 images using a custom-built CCD camera system (Pinkel *et al*., 1998) and carried out image and data analysis using UCSF SPOT (Jain *et al*., 2002). We used SPROC software to automatically filter the data to reject data points based on low DAPI intensity, low correlation between Cy3 and Cy5 within each segmented spot and low reference/DAPI signal intensity. We declared as missing observations with no replicates or with standard deviation of the replicates greater than 0.2. We screened out clones for the following reasons: data were missing in more than 15% of the samples, ratios on the clones had shown a median absolute value >0.2 in the normal samples, clones were not mapped on the genome sequence, or they were known as common copy number polymorphisms. For each tumor, we plotted the data in genome order as the mean $\log_2$ratio of the replicate spots for each clone normalized to the genome

median $\log_2$ratio. Array data are available in Supplementary Table B.

Statistical analysis

To enumerate genomic aberrations in tumors, we estimated the experimental variability of each CGH profile (s.d.) by taking the median of the median absolute deviations of the measurements on clones with the same copy number in that profile (Fridlyand *et al*., 2004). We declared a clone gained (lost) if its absolute value exceeded 2.5 times the s.d. for a given profile.

We used the Fisher exact test to test for independence of *TP53* mutation status with *EGFR* and *CCND1* amplifications and with the cluster assignments. For these analyses, we identified high-level amplifications by considering the magnitude and the width of the peak (Fridlyand *et al*., 2004). When comparing groups of tumors with or without *TP53* mutation, we tested for differential copy number at each clone on the array using a *t*-statistic with pooled variance. To assess significance for individual clones, we obtained the maxT-adjusted permutation-based *P*-values (Westfall and Young, 1993). We declared a clone to have a significantly different copy number between the two groups if its adjusted *P*-value was less than 0.05. Thus, there is less than 5% chance of obtaining one false positive result or more.

Quantitative RT–PCR

We isolated RNA from consecutive sections of formalin-fixed paraffin-embedded tumor resection or biopsy specimens (Macabeo-Ong *et al*., 2003) and performed real-time quantitative RT–PCR as described previously (Neve *et al*., 2002) in the UCSF Comprehensive Cancer Center Genome Analysis Shared Resource Facility. We used ABI Assays-on-Demand Expression assays. The specific assays used for each gene are provided in Supplementary Table A Expression levels were determined relative to expression of *GUSB* in each sample. Gene expression levels in tumors were compared to the mean expression levels of three normal tissue samples. We observed little variation in expression among the normal samples (Supplementary Table A). For most genes, twofold differences in expression in tumors relative to normals were greater than two s.d.'s above or below the mean of the expression in normals.

TP53 *sequencing*

We amplified exons 5–8 of *TP53* from genomic DNA of 75 tumors (23 tongue, 16 buccal mucosa, 17 floor of mouth, 19 gingiva) and carried out cycle sequencing as described previously (Moore *et al*., 2000) using modified primer sequences for exons 7 (forward: 5′-TGCCAC AGGTCTCCCCA-3′ and reverse: (5′-ATGGAAGAAAT CGGTAAGAGGTG-3′) and 8 (forward: 5′-CCTTACTGC CTCTTGCTTC-3′ and reverse: 5′-CATAACTGCACCCTT GGTC-3′).

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)