

Allele loss on chromosome 3 in squamous cell carcinoma of the head and neck correlates with poor clinical prognostic indicators

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Abstract. Cytogenetic studies in squamous cell carcinoma of the head and neck (SCCHN) have identified a clustering of breakpoints in a number of chromosomes, including chromosome 3. We have undertaken a loss of heterozygosity analysis (LOH) of 36 SCCHN and six solid tumours which were not squamous cell, and their respective normal specimens, using a bank of microsatellite markers, with the aim of identifying specific sites of frequent loss on chromosome 3. The analysis was undertaken with 12 microsatellite markers, 10 of which are on the p arm of chromosome 3. Allelic loss greater than 10% was seen at four sites; D3S1269 (13%), D3S1079 (23%), D3S659 (23%) and D3S1293 (31%). None of this series of tumours showed loss of the whole chromosome, however 47% of the tumours analysed had LOH at one or more loci. The highest incidence of LOH was found at D3S1293 in the 3p24-p25 region. The second highest region with LOH was found at D3S1079 and D3S659 at 3p13. The remaining markers telomeric and centromeric to these two regions were found to have a LOH of less than 10%. Furthermore, we found a strong association between loss of one marker on chromosome 3 in these SCCHN and poor clinical prognostic indicators; such as site, pathological differentiation, positive nodes at pathology ($p < 0.05$) and TNM status ($p < 0.05$). This study has identified two regions in SCCHN that are most likely to be important in the development of squamous cell carcinoma of the head and neck at 3p24-p25 and 3p13 and may indicate sites of novel tumour suppressor genes in this disease.

Introduction

Loss of heterozygosity (LOH) for polymorphic DNA markers have already provided a wealth of information on

chromosomal imbalances which would have been difficult if not impossible to obtain from cytogenetic evidence (reviewed by Lasko *et al*; 1). Specific sites of frequent allele loss has been described in a number of malignancies such as; carcinomas of the colon (2,3), ovarian (4-6), colorectal, bladder (7-8), and breast carcinomas (9) and tumour suppressor genes (TSG) have been suggested to be involved at many of these regions.

Cytogenetic studies in squamous cell carcinoma of the head and neck SCCHN has identified a clustering of breakpoints in a number of chromosomes, including chromosome 3. Jin *et al* (10) found a high incidence of chromosomal breakpoints at 1p22 and 11q13, whereas other groups have found 1p36, 9q32, 11q23, (11) 3p, 8q, 10p, 18q, (12), 3p13-14, 8p21-23 and 18q21-23 (Van Dyke DL, Worsham MJ, Benninger MJ and Carney TE: 5th International Workshop on chromosomes in solid tumours, Arizona, 1993). However, there are many limitations to the interpretation of cytogenetic analysis of head and neck cancers as the majority of the chromosomes have been found to be involved (10,11). Latif *et al* (13) have used a bank of restriction fragment length polymorphism (RFLP) probes, and generated a 3p allelotype for 18-26 SCCHN cell lines. Their observations indicated that there was a commonly deleted region on 3p which lies telomeric to 3p14 and centromeric to 3p25. Furthermore, in a cytogenetic analysis of 15 laryngeal tumours, a deletion in 3p was found in 60% of the cases analysed (14). This region of the chromosome has received a great deal of interest in a number of malignancies resulting in the mapping of the Von Hippel-Lindau TSG to 3p25-26, small cell lung carcinoma genes to 3p23-p21, non-small cell carcinoma gene to 3p14, uterine cervix gene to 3p14-p21 and renal cell carcinomas to 3p14.2 (15).

The development of banks of microsatellite markers has now advanced the analysis of LOH studies (16,17). The microsatellites constitute one of the most abundant classes of repetitive DNA families in the human genome with approximately 100,000-150,000 (CA)_n repeats, and have been used extensively in linkage analysis (18) and are probably more sensitive than RFLP analysis.

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We have investigated chromosome 3 in SCCHN with 12 microsatellite markers in order to determine the incidence of LOH especially on the p arm of chromosome 3 and evaluate the results with the clinicopathological data.

Materials and methods

Forty-two tumour specimens were collected from patients with carcinomas of the head and neck by the Royal Liverpool Hospital, Department of Otorhinolaryngology (36 SCCs; hypopharynx (18), larynx (11), oropharynx (4) oral (2), and 2 basal cell carcinomas, a malignant salivary tumour, a Warthins tumour, a chondroma and a neurosarcoma were selected for this study. Tumour samples obtained from surgical specimens were frozen in liquid nitrogen and stored at -70°C .

Frozen sections were taken from representative parts of the tumour and stained with haematoxylin and eosin and the percentage of tumour to normal tissue was recorded. All tumour specimens used in this study contained more than 50% tumour tissue compared with normal tissue. Clinicopathological and survival data was available for the majority of the patients investigated in this study.

DNA extraction. Genomic DNA was extracted from tumour specimens using the Nucleon II DNA extraction kit from Scotlab, following the manufacturers instructions. Genomic DNA samples were stored at 4°C .

Microsatellite repeat primers. The markers used in this study are shown in Table I. and their chromosomal location in Fig. 1. The Weissenbach markers have been localised on the genetic map using all available published data to date. The microsatellite primers D3S1261, D3S1266, D3S1269, D3S1289, D3S1293, D3S1340 were obtained from Research Genetics, USA; D3S966, D3S1067, D3S1079 from the UK Human Genome Mapping Project; D3S1284 from Professor P. Humphries University of Dublin; D3S659, RHO from Dr Pamela H. Rabbitts, University of Cambridge. The annealing temperature for each marker is given in Table I. Details of the microsatellite primers are available in the Genome Data Base, John Hopkins University, USA and from the UK Human Genome Mapping Project Research Centre, and Jones *et al.* (19).

LOH analysis. PCR reactions were performed in a 50 μl reaction volume and contained 200 ng of genomic DNA, 500 μM dNTP, 10 pmoles of each forward and reverse microsatellite primers, 0.5 units of Taq DNA polymerase (Advanced Biotechnologies), 5 μl of 10X PCR Buffer (670 mM Tris.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7mg/ml BSA; 100 μM β -mercaptoethanol; 1% (w/v) Triton X-100). The reactions were denatured for 4 minutes and the DNA was subsequently amplified for 25 cycles of 94°C for 30 seconds, the appropriate primer annealing temperature (Table I) for 30 seconds and 72°C for 1 minute. 10 μl of the amplified PCR reaction mixture was electrophoresed overnight on a 10% acrylamide gel at 20 mA for 2500V h. The acrylamide gels were silver-stained and the DNA bands observed over a light box.

Statistical analysis. Quantative data were analysed by X^2 or Fisher's exact test where appropriate. Survival curves were drawn up using the Kaplan-Meier product limit estimate (20). Differences between survival times were analysed by the log rank method (21).

Results

We analysed 36 SCCHN and 6 non squamous tumours from the head and neck regions for LOH at 10 different loci on chromosome 3p and 2 loci on 3q using PCR microsatellite pairs. The LOH results are shown in Table II for all the patients (Pts) analysed, and the LOH frequencies at each locus are shown in Table III. Loss of heterozygosity is demonstrated in Pt. 343 at the D3S1293, D3S966, D3S1067 and D3S1079 loci; heterozygous at the D3S1304 and D3S1269 loci and homozygous at the RHO locus (Fig. 2).

Allele loss greater than 10% was seen at four loci, D3S1269 (13%, 4/30); D3S1079 (23% 5/22); D3S659 (23%/6/26) and D3S1293 (31% 9/29). In this series of SCCHN, none of the tumour specimens showed whole chromosome loss, however 47% (17/36) of the tumours analysed had LOH at one or more loci.

The highest incidence of loss of heterozygosity was found at D3S1293 (31%) in the 3p24-25 region. No LOH was found at D3S1266 in 36 specimens of SCCHN, which has been localised to the 3p24 region centromeric to D3S1293. The Weissenbach *et al.*, 1992 map indicates a 9cM distance between these two microsatellite markers. It is improbable that there is a large deletion at this site, as only two of these patients (327, 341) had LOH at D3S1289, which is 30cM centromeric to D3S1293. It is of note that in five cases (Pts. 217, 327, 338, 343, 347) there is also loss at D3S1079 as well as D3S1293 and both of these markers are located at 3p13. Furthermore, the D3S1304 marker is 19cM telomeric to D3S1293 and only 7% of the SCCHN specimens had LOH at this locus.

The clinicopathological data has been analysed in conjunction with the LOH results, for site, TNM stage, histological differentiation, positive nodes at pathology and survival. A clear association has been found between LOH on chromosome 3 and squamous cell carcinoma (SCC) at the hypopharyngeal site 58% (11/19), compared to 36% (4/11) LOH at the laryngeal site. Furthermore, an association between LOH on chromosome 3 and a poor clinical prognosis as judged by the level of differentiation, positive nodes at pathology and TNM staging has been found (Tables IV and V).

Analysis of LOH on chromosome 3 and histological differentiation demonstrated an increase in the incidence of allelic loss with the level of differentiation; well differentiated 20%, moderately differentiated 48% and poorly differentiated squamous cell carcinoma 83%, but this was not found to be statistically significant as the numbers of patients in certain groups were small ($p > 0.05$). However, a dramatic increase in the number of positive nodes at pathology was seen to correlate with LOH; SCCHN with no lymph nodes had 21% LOH, whereas SCCHN with positive lymph nodes had 63% LOH ($p < 0.05$). This trend with LOH on chromosome 3 with poor prognostic factors is further supported by an association with TNM staging of the tumours. Stages I, II, III had 33%

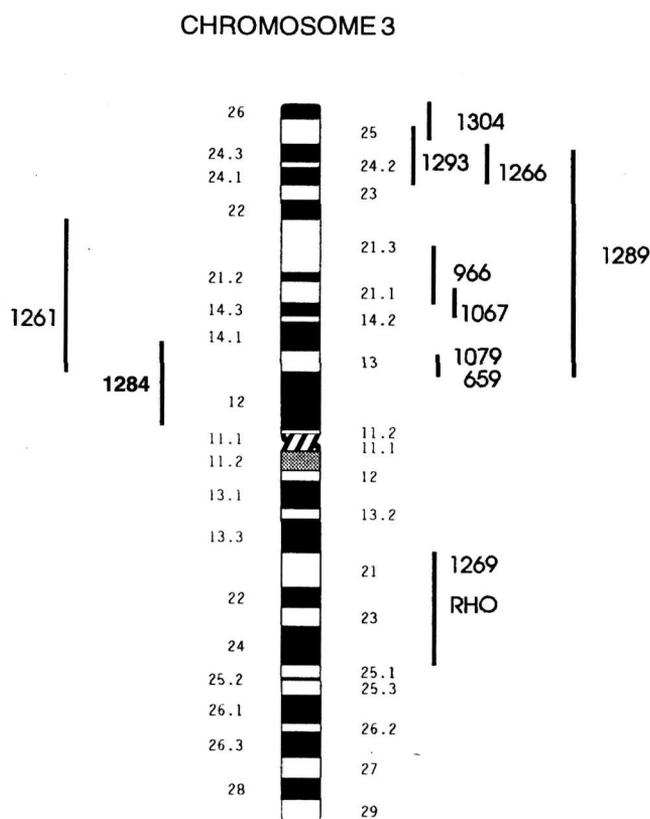


Figure 1. A karyogram of chromosome 3. The microsatellite marker loci are shown adjacent to their approximate map position based on previously published data and the LOH analysis in this study.

(7/22) LOH compared to Stage IV, which had 77% (10/13) LOH ($p < 0.05$). No correlations with survival data was found, however the follow-up for a number of these patients has been less than six months.

Six other solid tumours of the head and neck region which were not squamous cell were also investigated and only one of these, a BCC had LOH at one locus (results not shown).

Discussion

In this study we have investigated loss of heterozygosity in 36 SCCHN tumour specimens on chromosome 3 using a bank of microsatellite markers, the majority of which are on the 3p arm. We have identified one region with a high LOH (31%) using the D3S1293 marker which is located in the 3p24-25 region. In view of the fact that the markers centromeric (D3S1266) and telomeric (D3S1304) to D3S1293 were found to have low LOH, argues strongly in favour of this locus being a possible site of a novel tumour suppressor gene in SCCHN.

A region on chromosome 3p, 3p14 (D3S3) to 3p25 (RAF1) has been previously identified in SCCHN cell lines to have high allelic loss (13). These authors used novel statistical analysis to determine the importance of LOH on 3p. They generated an SCCHN alleotype of 3p using a number of SCCHN cell lines with RFLP markers and then

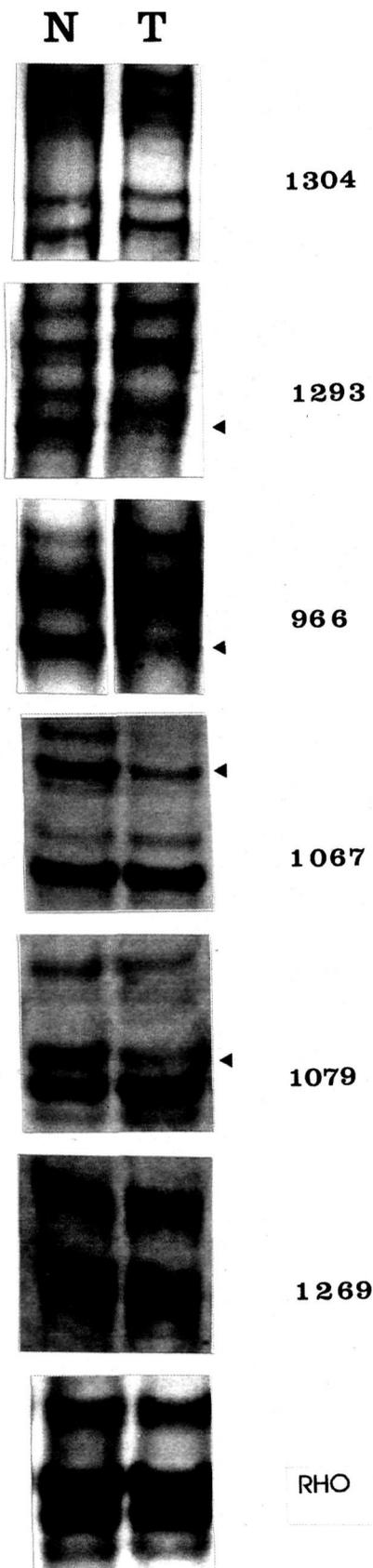


Figure 2. LOH on chromosome 3, detected by PCR analysis of microsatellite loci in tumour 343, seven of the microsatellite markers are shown in this figure; D3S1304 heterozygous, D3S1293 LOH, D3S966 LOH, D3S1067 LOH, D3S1079 LOH, D3S1269 heterozygous, RHO homozygous. Note the examples of a heteroduplex band (top band) and the stutter bands seen for both the alleles in D3S1067.

Table I. Microsatellite repeat primers used in this study of loss of heterozygosity on chromosome 3 in head and neck squamous cell carcinomas.

Microsatellite primers	Map position	Futher information on map position*	Annealing temperature
D3S1304 ^W	3p25-pter	19 cM telomeric from D3S1293	55°C
D3S1293 ^W	3p24-p25	located telomeric to pBH302-H/THRB. 9 cM from D3S1266	55°C
D3S1266 ^W	3p24	in region of CRI-R59-M/D3S12	55°C
D3S1289 ^W	[3p24]-[3p21.2-p13]	located midway between CRI-R59-M/3S12 and CRI-R95-M/D3S13 21 cM centromeric from D3S1266	62°C
D3S1261	3p21.2-p13		63°C
D3S966	3p21		60°C
D3S1067	3p14.3-p21.1		60°C
D3S1079	3p13		60°C
D3S659	3p13		50°C
D3S1284	3p12-p13		58°C
D3S1269 ^W	3q	in the region of CRI-C17-M/D3S20 7cM from RHO	57°C
RHO	3q21-q24		50°C

*Information taken from Weissenbach *et al* 1992; ^WWeissenbach marker set (17).

compared the average heterozygosity computed for these loci in randomly chosen individuals from a natural population, with the observed heterozygosity of the tumour cell DNAs. They firstly deduced that there was a general LOH in the SCCHN cell lines, and then estimated the loss for each individual locus, thereby delimiting the commonly deleted region. A synopsis of their findings are shown in Table VI. It is of note that they found the probability of the markers at 3p24-25, D3S588 and THRB in the SCCHN cell lines (having the same heterozygosity as in normal individuals) to have values of 4×10^{-10} and 0.0003, respectively. Our results substantiate these findings, as the D3S1293 (3p24-25) marker is located telomeric to THRB on the Weissenbach map, therefore it is most likely closely linked to the D3S588 RFLP marker. In addition, a number of markers centromeric to THRB and telomeric to D3S3 also had high probabilities of loss in tumour DNA and Latiff *et al* (13) defined the whole 3p13-p25 region of particular importance in SCCHN. The results reported in this study are in agreement with the findings of Latiff *et al*, as our LOH data indicates that there are specific LOH regions at 3p24-25 and also at 3p13. Both of the markers D3S1079 and D3S659 located at 3p13 demonstrated a LOH of 23%. However, the intervening markers between D3S1293 (3p24-25) and the 3p13 markers all had LOHs of less than 10%, which may be considered background LOH in tumour specimens. Even though many

of the Weissenbach markers have not been precisely located to the genetic map, Fig. 1 provides a reasonable assessment of their relative positions based on previously published data and the LOH analysis results in this study.

A further argument for the presence of two putative TSGs on 3p comes from our clinical correlations with LOH analysis on chromosome 3. The clinicopathological parameters used to assess patients outcome were recorded for all of the patients investigated in this study. The most important are site, pathological differentiation, positive nodes at pathology and the TNM status of the tumour at diagnosis. All of these parameters were found to be associated with LOH on chromosome 3. SCCHN at the hypopharyngeal site constituted the largest group of patients studied, of whom 58% (11/19) had lost one or more markers on chromosome 3, compared with the laryngeal tumours which had 36% LOH (4/12) on one or more markers on chromosome 3. Furthermore, the clinical prognosis of SCC at the hypopharyngeal site is considered to have a worse prognosis than the larynx (22).

The candidate genes for 3p24 and 3p13 sites are listed in Table VII, these include CCK, ACT14, IL5RA, RAF1, FRA3A, IMPDH2, THRB, RARB, TOP2B, and RAB5 in the 3p24-pter region. Recent evidence indicates that there may be as many as three TSGs on the p arm of chromosome 3. Hibi *et al* (23) have shown that there are three distinct

Table II. An analysis of 36 SCCHN by PCR microsatellite primers.

	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
	114	128	184	192	218	224	227	302	310	313	314	324	325	329	336	338	340	341	346
D3S1304	○	○	○	○	-	○	-	○	-	○	∅	-	○	●	○	○	-	●	○
D3S1293	○	○	●	○	○	○	●	○	∅	∅	○	∅	○	●	○	●	-	●	○
D3S1266	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
D3S1289	○	○	○	○	∅	○	○	○	●	○	○	○	∅	○	○	○	○	○	○
D3S1261	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	∅	∅	○
D3S966	∅	○	●	○	○	○	○	○	∅	○	○	∅	∅	○	○	○	○	○	○
D3S1067	∅	○	-	∅	∅	○	-	○	-	∅	○	○	○	∅	○	○	○	∅	-
D3S1079	∅	∅	∅	-	○	○	-	○	○	○	○	○	*	∅	∅	●	○	∅	∅
D3S659	○	○	∅	●	○	○	-	○	○	○	●	●	*	○	○	○	-	-	●
D3S1284	○	○	-	-	-	○	-	○	-	○	○	○	○	○	○	○	○	○	○
D3S1269	○	○	●	-	-	○	●	○	○	○	○	○	○	○	○	○	○	○	○
RHO	-	∅	-	○	-	○	-	○	-	○	∅	∅	○	∅	∅	∅	-	○	∅

	L	L	L	L	L	L	L	L	L	L	L	M	M	M	M	O	O
	41	76	132	202	213	215	217	223	225	318	347	315	327	342	348	201	343
D3S1304	∅	-	-	○	-	○	-	○	○	○	○	-	○	○	○	-	○
D3S1293	○	●	○	-	○	○	●	○	-	○	●	○	●	○	∅	○	●
D3S1266	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
D3S1289	○	○	○	○	○	○	∅	○	○	○	○	○	●	○	○	○	○
D3S1261	○	●	○	○	○	○	○	○	○	○	○	○	∅	○	○	○	○
D3S966	○	○	○	○	○	○	∅	○	○	○	○	∅	∅	○	○	∅	●
D3S1067	∅	-	-	○	○	○	∅	∅	-	○	○	∅	○	○	-	○	●
D3S1079	○	-	-	○	○	○	●	∅	○	○	●	○	●	○	○	∅	●
D3S659	○	-	●	○	○	○	●	∅	-	○	○	○	∅	○	○	○	∅
D3S1284	○	-	○	∅	○	○	○	∅	-	○	○	○	○	○	○	○	○
D3S1269	○	∅	-	-	○	○	●	○	-	○	○	○	○	○	○	○	○
RHO	○	-	-	○	-	○	-	○	○	○	∅	-	∅	○	○	-	∅

The symbols used are: ●, constitutional heterozygosity with loss of heterozygosity; ○, constitutional heterozygosity with no loss of heterozygosity; ∅, homozygous; not done/not determined; O, oropharynx; H, hypopharynx; L, larynx and M, oral cavity.

Table III. Loss of heterozygosity on chromosome 3 in head and neck squamous cell carcinomas.

Chromosome location	Microsatellite primers	Number of patients analysed	Allelic loss/ informative cases (% LOH)
3p25-pter	D3S1304	24	2/23 (7)
3p24-p25	D3S1293	33	9/29 (31)
3p24	D3S1266	36	0/36 (0)
3p24-p13	D3S1289	36	3/33 (9)
3p21.2-p13	D3S1261	36	2/33 (6)
3p21	D3S966	36	2/28 (7)
3p14.3-p21.1	D3S1067	28	1/17 (6)
3p13	D3S1079	31	5/22 (23)
3p13	D3S659	30	6/26 (23)
3p12-p13	D3S1284	29	0/27 (0)
3q	D3S1269	31	4/30 (13)
3q21-q24	RHO	24	0/14 (0)

Table IV. Loss of heterozygosity on chromosome 3 with TNM staging.

Stage	Loss of one or two markers	Loss of 3 or more markers	No loss	LOH/ Heterozygous	Percent
I	2	0	5	2/7	28
II	0	0	1	0/1	
III	3	2	10	5/14	33
IV	7	3	3	10/13	77

Stages I, II, III with IV, $p = 0.006$

Table V. Loss of heterozygosity on chromosome 3 with histological differentiation and positive nodes at pathology.

	Loss of one or more markers on chromosome 3	No loss	Percentage
Histology:			
well differentiated	1	4	20
moderately differentiated	10	11	48
poorly differentiated	5	1	83
			($p > 0.05$)
positive nodes at pathology:			
no nodes	3	11	21
positive nodes	10	6	63
			($p < 0.05$)

Table VI. Chromosome 3p deletions in head and neck cell lines.^a

Location on 3p	Locus	Probability*
3p25	D3S571	0.0038
3p25	RAF	0.0009
3p24-25	D3S588	4×10^{-10}
3p24	THRB	0.0003
3p21-p24	D3S734	4×10^{-5}
3p21-p24	D3S232	0.0022
3p21	D3F15S2	1×10^{-7}
3p14-p21	D3S32	8×10^{-5}
3p14.2-p21	D3S2	9×10^{-6}
3p13	D3S642	4×10^{-5}
3p13	D3S3	0.1307

^adata from Latif *et al.* (13); Probability that 3p loci in tumour DNA have the same heterozygosity as that of the normal population. *all significant at the 0.0023 level.

regions in lung cancer, 3p25, 3p21.3 and 3p14. Moreover, the 3p14 region has been further defined in lung cancer by Yamakawa *et al.* (24), and the 3p13-p21.1 region has been

identified in uterine-cervical cancer (25) as a site of a putative TSG.

This study has identified two regions in SCCHN that are probably important in the development of squamous cell carcinoma of the head and neck (3p24-p25, 3p13), however, a larger number of patients will have to be studied with a bank of specific markers in these regions to determine their importance.

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Table VII. Candidate genes on chromosome 3p.

Chromosomal location		Name of gene locus
3pter-p21	CCK	Cholecystokinin
3pter-q21	ACTL4	Actin-like 4
3p26-p24	IL5RA	Interleukin 5 receptor alpha
3p25	RAF1	Murine leukemia viral (<i>v-raf-1</i>) oncogene homolog 1.
3p24.2-p21.2	IMPDH2	Inosine monophosphate dehydrogenase 2
3p24.1-p22	THRB	Thyroid hormone receptor beta (oncogene <i>erbA2</i>)
3p24	RARB	Retinoic acid receptor beta
3p24	TOP2B	Topoisomerase 2 beta
3p24-p22	RAB5	<i>Ras</i> superfamily member
3p23-p22	ACAA	Acetyl coA acyltransferase
3p23-p22	GLB1	Galactosidase beta 1
3p22-p21	ZNF35P	Zinc finger protein 35 pseudogene
3p21.3	ZNF52	Zinc finger protein
3p21.3	ZNF64	Zinc finger protein
3p21.2-p21.1	ITIH1	Inter-alpha (globulin) inhibitor, H1 polypeptide
3p21.2-p21.1	ITIH3	Pre-alpha (globulin) inhibitor, H3 polypeptide
3p21.1	ACY1	Aminoacylase 1
3p21-p14	PTPRG	Protein tyrosine phosphatase, receptor alpha.
3p14-q21	SRML2	Spermidine synthetase-like 2

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