**Abstract**

**Introduction:** To understand the role of two interacting proteins LIMD1 and pRB in development of head and neck squamous cell carcinoma (HNSCC), alterations of these genes were analyzed in 25 dysplastic head and neck lesions, 58 primary HNSCC samples and two HNSCC cell lines.

**Methods:** Deletions of LIMD1 and RB1 were analyzed along with mutation and promoter methylation analysis of LIMD1. The genotyping of LIMD1 linked microsatellite marker, hmlimD1, was done to find out any risk allele. The mRNA expression of LIMD1 and RB1 were analyzed by Q-PCR. Immunohistochemical analysis of RB1 was performed. Alterations of these genes were correlated with different clinicopathological parameters.

**Results:** High frequency [94% (78/83)] of LIMD1 alterations was observed in the samples studied. Compare to frequent deletion and methylation, mutation of LIMD1 was increased during tumor progression ($P = 0.007$). Six novel mutations in exon1 and one novel intron4/exon5 splice-junction mutation were detected in LIMD1 along with a susceptible hmlimD1 (CA)$_{20}$ allele. Some of these mutations [42% (14/33)] produced non-functional proteins. RB1 deletion was infrequent (27%). Highly reduced mRNA expression of LIMD1 (25.1 ± 19.04) was seen than RB1 (3.8 ± 8.09), concordant to their molecular alterations. The pRB expression supported this data. Tumors with LIMD1 alterations in tobacco addicted patients without HPV infection showed poor prognosis. Co-alterations of these genes led the worse patients’ outcome.

**Conclusions:** Our study suggests LIMD1 inactivation as primary event than inactivation of RB1 in HNSCC development.

**Introduction**

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy, accounts for 30-40% of all cancer types in Indian subcontinent [1]. Tobacco, betel nut leaf quid, alcohol, HPV-16/18 infection are well recognized carcinogenic risk factors for development of this cancer [2]. Despite significant progress in understanding molecular genetic events underlying the development of HNSCC, details mechanisms still remain unknown [3,4]. Suppression of tumorigenicity of oral cancer cell lines following introduction of chromosome 3p in microcell hybrid system, suggested the presence of at least one tumor suppressor gene (TSG) in this chromosome associated with HNSCC development [5]. Our previous study in HNSCC of Indian patients showed high frequency of loss of heterozygosity (LOH) in chromosomal (chr.) 3p21.31 region and its association with development of early dysplastic lesions [6]. Among the multiple TSGs localized in chr.3p21.31, our recent study demonstrated one of the candidate TSGs, LIMD1 alteration (deletion/methylation) was significantly associated with mild dysplastic lesions of head and neck [7]. Downregulation of this gene observed in HNSCC and lung cancer [7,8]. A recent study emphasized LIMD1 as a critical TSG showing frequent downregulation in expression due to genetic and epigenetic modification in human lung cancer [9]. But no coding region mutation of this
gene was observed in lung cancer. Also a polymorphic dinucleotide cytosine-adenine [d(CA)] microsatellite repeat, hmlimD1 (Accession number EU125867) was located at 15 bp upstream of LIMD1 gene [7]. Susceptibility allele of this gene, if any, for HNSCC development was unknown. LIMD1 has 8 exons and encodes a 676 amino acid protein, with a leucine-rich nuclear export signal (NES) in its N-terminal Pre-LIM domain and in C-terminus harboring three LIM domains having nuclear localizing properties (NLS) [8-10]. It is a ZYXIN family protein, having tandem zinc fingers in its LIM domains facilitating protein-protein interactions [11]. LIMD1 was reported to inhibit cell growth and metastases, partly mediated through either an interaction of its N-terminal LEM domain (amino acid 18-68) with barrier-to-autointegration (BAI), a component of SWI/SNF chromatin-remodeling protein, or through interaction of its part of proline-serine rich domain (amino acid 326-608) with C-terminus of retinoblastoma protein, pRB (amino acid 763-928) followed by transcriptional repression of E2F target genes [8]. This might be due to the stabilization of pRB-E2F interaction. The retinoblastoma gene, RB1 was reported to be infrequently altered in HNSCC [12,13]. Our previous study showed RB1 gene deletions were mainly associated with later stages in HNSCC development [14,15]. However, alterations of LIMD1 and RB1 were not screened in same set of samples to understand their association together in development of the disease.

Thus in this study attempts have been made to analyze the alterations of LIMD1 and RB1 in 25 dysplastic head and neck lesions, 58 primary HNSCC samples and two HNSCC cell lines. We have screened LIMD1 mutation in the entire exon1 (1429 bp) and exon5 along with RB1 deletion and its protein expression (by immunohistochemistry, IHC) in the same set of samples. The frequency of LIMD1 mutation was then compiled with our previously reported [7] of its deletion, promoter methylation and mutation (in the SNP rs267236 site in exon1) frequencies in these samples for clinicopathological correlation. The alterations of LIMD1 were also correlated with RB1 alterations. A population-based case-control study was performed to find out any risk allele of hmlimD1. Our data demonstrated that inactivation of LIMD1 was primary event than RB1 in development of HNSCC.

Materials and methods

Patients, controls and cell lines
A total of 37 dysplastic lesions and 110 HNSCC tumors and their matched normal tissues were collected from 147 unrelated individuals after obtaining informed consent from patients according to hospital authorities of Chittaranjan National Cancer Institute and Cancer Center & Welfare Home, Kolkata, India. All tumors were graded and staged according to UICC TNM classification [16]. Freshly operated tissues were taken for isolation of DNA/RNA and immunohistochemical analysis. Among these samples deletion/methylation/mutation analysis of the genes was performed in 25 dysplastic lesions (mild, n = 3; moderate, n = 9; severe, n = 13), 58 HNSCC samples (stageI, n = 8; stageII, n = 11; stageIII, n = 21; stageIV, n = 18) and two HNSCC cell lines Hep2, UPCI: SCC084. However, the total samples pool was used for case-control study. Table 1 presented clinicopathological information of patient (n = 147) and unrelated controls (n = 187) having no previous and present history of HNSCC. Blood samples were collected from controls with informed consent, and Institutional Ethical Board approved the study. All patients and controls were an age and sex frequency-matched ethnically similar caste population from eastern India. Two HNSCC cell lines, Hep2 and UPCI: SCC084 were obtained from National Centre for Cell Sciences, Pune, India and from Prof. Susanne M. Gollin, University of Pittsburgh, USA, respectively.

Microdissection and DNA Extraction
Cryosections (5 μm) were microdissected under dissecting microscope (Leica MZ16, Germany) using surgical knives to remove contaminant normal cells. Controls’ blood and samples containing >60% tumor cells were taken for DNA isolation by phenol/chloroform extraction [17].

Mutation analysis
LIMD1 was screened for mutation in 25 dysplasias, 58 HNSCC samples and the two HNSCC cell lines by single strand conformation polymorphism (SSCP) analysis using [α-P32] dCTP [14]. For mutation analysis six sets of primers were designed to amplify whole exon1 and one set for the exon5 including their respective intron/exon borders (see Additional file 1: Table S1). In these samples deletion and promoter methylation of LIMD1 have already been reported [7], along with mutation status in exon1 at the SNP rs267236 site using LIMD1 Exon1.6 primer set (see Additional file 1: Table S1) in the 77/85 samples. Electrophoresis was done in 6% non-denaturing polyacrylamide gel with 10% glycerol at 2W for overnight and autoradiographed on X-ray film (Kodak, USA). Samples showing abnormal band shifts were sequenced using Genetic Analyzer (PE Applied Biosystems Inc, USA).

Deletion analysis
Deletion mapping of RB1 was done in the same set of 25 dysplasias, 58 HNSCC samples and two HNSCC cell lines (as mentioned in Mutation analysis) using one intragenic microsatellite marker, D13S153 (intron2 of RB1, 47.8 Mb from p-ter) [15].
Genotyping of hmlimD1 microsatellite marker

Genotyping of hmlimD1 was carried out by amplifying (CA)n repeat in a standard polymerase chain reaction (PCR) using 5'-P32] ATP labeled forward primer. PCR products were electrophoresed on 7% denaturing polyacrylamide sequencing gel and autoradiographed [18]. Signal intensities of radio-labeled products were measured by densitometric scanner (Bio-Rad, USA). Amplified fragments ranged in size from 187-216 bp, depending on the number of CA repeats within amplified region. Representative homozygote for (CA)17, (CA)19, (CA)20, (CA)21, (CA)24, and (CA)32 genotypes were sequenced using Genetic Analyzer (PE Applied Biosystems Inc, USA) to validate CA repeats number.

mRNA expression analysis

The mRNA expression of LIMD1 and RB1 genes was analyzed by quantitative RT-PCR in paired primary HNSCC samples (n = 24) and two HNSCC cell lines using primers mentioned in Additional file 1: Table S1. The LIMD1 mRNA expression was already reported in 11 of the 24 HNSCC samples and the two cell lines [7]. Total RNA was isolated from the samples using TRIzol reagent (Invitrogen, USA) and complementary-DNA (cDNA) was synthesized using Random hexamer (Invitrogen, USA) and M-MuLV Reverse Transcriptase (Sibenzyme, Russia). Real-time quantification of LIMD1 and RB1 was performed in 40 cycles on an ABI Prism 7500 using Power SYBR Green PCR Master Mix (Applied Biosystems, USA).

Table 1 Characteristics of patients and controls

| Controls(187) | HNSCC & Dysplastic lesions(147) |
|---------------|----------------------------------|
| Sex*          | Sex*                             |
| Male, N(%)    | Male, N(%)                       |
| Female,(N%)   | Female,(N%)                      |
| Mean Age*     | Mean Age*                        |
| 157(83.9%)    | 110(74.9%)                       |
| 30(16.1%)     | 37(25.2%)                        |
| 45.5 ± 5.2    | 52 ± 6.4                         |

| HNSCC (110) | Oral Cavity tumors(85) |
|-------------|------------------------|
| Buccal Mucosa, N(%) | 32(29.1%) |
| Tongue, N(%) | 13(11.8%) |
| Cheek, N(%)  | 19(17.3%) |
| Lip, N(%)    | 3(2.7%)  |
| Tonsil, N(%) | 4(3.6%)  |
| Palate, N(%) | 2(1.8%)  |
| Alveolus, N(%)| 11(10%)  |
| Vocal cord, N(%) | 1(0.9%) |
| Laryngeal Tumors(12) & | Larynx, N(%) |
| Nasopharyngeal Tumor(3) | 12(10.9%) |
| Nassopharynx, N(%) | 3(2.7%)  |
| Orofacial Tumors(10) | Maxilla, N(%) |
| Mandible, N(%) | 5(4.5%)  |
| Histopathology(110) | StageI |
| StageII | 15(14%)  |
| StageIII | 30(27%) |
| StageIV | 34(31%)  |
| StageV | 31(28%)  |
| Dysplastic lesions (37) | Buccal Mucosa, N(%) |
| Histopathology(37) | Mild Dysplasia |
| Moderate Dysplasia | 5(13.5%)  |
| Severe Dysplasia  | 19(51.4%) |
| 37(100%)       | 13(35.1%) |

* Difference in sex distribution: Control vs. HNSCC, P = 0.04,

# Mean age difference: Control vs. HNSCC, P = 0.01.
in a final volume of 25 μl human β2-microglobulin gene (B2M) was used as control. Each sample was loaded in triplicate. Relative level of gene expression was determined by comparative threshold cycle (ddCt) method [19] after normalization against B2M.

**Immunohistochemical analysis**

pRB expression was determined by immuno-staining in 5 dysplasias, 15 HNSCC samples and the two cell lines. Paraffin sections of primary tissues and cover slip cultures of the cell lines were reacted with 1:100 dilution of primary antibodies (rabbit polyclonal IgG, sc-7905, for pRB [M-153] Santa Cruz, USA, raised against amino acids 769-921 from C-terminus of pRB). HRP-conjugated (sc-2004 with 1:500 dilutions) and FITC-tagged (sc-2012 with 1:100 dilutions) goat anti-rabbit secondary antibodies (Santa Cruz, USA) were used for the primary tissues and cell lines respectively. For permanent staining of the primary tissues, the slides were developed using 3, 3′diaminobenzidine as the chromogen and counterstained with hematoxylin. The staining intensity (1 = weak, 2 = moderate, 3 = strong) and the percentage of positive cells (<1 = 0, 1-20 = 1, 20-50 = 2, 50-80 = 3 and >80 = 4) were detected by two observers independently and by combining the two scores.

**Detection of HPV-16 and HPV-18**

Presence of HPV in HNSCC was detected by PCR using primers (MY09 and MY11) from consensus L1 region followed by typing of HPV 16/18 in L1 positive samples (oral cavity n = 2). Post-operative overall survival was measured from date of surgery to date of last follow-up or death (upto 5 years). P-values were evaluated by log-rank test for censored survival data. Significance and relative risk of various potential prognostic factors was evaluated by Cox proportional hazards model and the hazard ratio (HR) for each parameter with 95% confidence interval (CI) in a multivariate fashion were estimated to predict joint impact of several prognostic factors on overall survival of patients with oral cavity cancer. Analysis of other sites in HNSCC was excluded due to small sample size. All statistical analysis was performed using EpilInfo 6.04b, SPSS 10.0 (SPSS Inc. Chicago, IL, USA).

**Results**

**Alterations of LIMD1**

To prove LIMD1 as a candidate TSG, mutation in exon1 and exon5 of this gene was screened in 83 of primary head and neck lesions (25 dysplasias and 58 HNSCC) and two HNSCC cell lines. The deletion and promoter methylation status of LIMD1 were screened in these sample previously [7]. Six novel mutations were observed in exon1 (Fig 1A-E) and one was in splice- junction of intron4/exon5 (C deletion 3nt upstream of exon5; Fig 1F, Table 2). Among the mutations in exon1, one transversion with aminoacid changes at 1003C→A (Pro335Thr; Fig 1E) and one was in splice- junction of intron4/exon5 (C deletion 3nt upstream of exon5; Fig 1F, Table 2). Among the mutations in exon1, one transversion with aminoacid changes at 1003C→A (Pro335Thr; Fig 1E), three transition with aminoacid changes at 470C→T (Ser157Phe; Fig 1A) and without aminoacid changes at 882T→C (Thr294Thr; Fig 1C) and 1068T→C (Gly356Gly) [7] and two frameshift mutations due to single nucleotide deletions at 660A (Fig 1B) and 967T (Fig 1D) were observed. Two transition mutations were overlapped with the two SNPs (rs267237 and rs267236) (see additional file 2: Figure S1 and additional file 3: Figure legend for Figure S1.txt). Frameshift mutations led to premature terminations of proteins (Table 2, Fig 2). About 40% (33/83) of the primary head and neck lesions showed mutations in at least one site. Majority of mutations were clustered in proline and serine rich domain of LIMD1 (Fig 2). No mutation was observed in Hep2 and UPCI: SCC084 cells. The status and pattern of LIMD1 alterations (deletion/methylation/mutation) in the 83 of primary head and neck lesions and the two HNSCC cell lines were presented in the additional files (see Additional file 4: Table S2 and Additional file 5: Table S3). Mutation frequency of LIMD1 was low (8%, 2/25) in dysplastic lesions, but significantly (P = 0.007) increased in stage (I+II) tumors (42%, 8/19) (Fig 3A). Moreover, significant association has been seen between LIMD1 mutation and its methylation in HNSCC samples (Table 3).

**Association of hmlim1 polymorphism with risk of HNSCC**

To find out LIMD1 susceptible allele, if any, associated with HNSCC risk, we analyzed allele polymorphism of hmlim1 microsatellite marker in population based case-control study. Age and sex distribution between the cases and controls were found to be statistically significant (Table 1). A total of 12 CA repeat alleles were observed in our study population, ranging from 9 repeats [(CA)9] to 38 repeats [(CA)38] (Table 4). Among them, alleles (CA)17
and (CA)_{19} were relatively common (>10% in frequency) in control population. However, in cases >10% allele frequency was seen in (CA)_{19}, (CA)_{20} and (CA)_{24} alleles. Overall, case-control difference in allele distribution was seen to be statistically significant ($P = 0.0021$). Comparing the frequency of each allele with that of all other alleles combined, case-control differences were significant for (CA)_{20} [$P = 0.000005$], (CA)_{32} [$P = 0.0082$] and borderline significance for (CA)_{24} allele [$P = 0.05427$]. The (CA)_{20} allele was over represented in cases, whereas (CA)_{17} and (CA)_{19} alleles were under represented in cases where >10% allele frequency of the alleles were seen. In comparison to homozygous alleles distribution among cases and controls, significant association of $[(CA)_{20}/(CA)_{20}]$ allele ($P = 0.00003$) was seen with the cases. Alleles (CA)_{32} and (CA)_{38} were rare in this study population.

**Association of LIMD1 alterations with RB1 deletion in HNSCC**

As LIMD1 was shown to interact with pRB [8], we studied the association of alterations of both genes. Compare to high LIMD1 alterations, RB1 deletion was very low (27%, 22/83) in the primary head and neck lesions (Fig 3B). Deletion frequency of RB1 was low in dysplastic lesions (8%, 2/25) and significantly ($P = 0.045$) increased to stage (I+II) tumors (32%, 6/19) consistence with our previous findings [13,14] (Fig 3B). No association between LIMD1 alterations and RB1 deletion was observed, however corelation of both genes was high (33%, 13/39) in the stage III+IV of head and neck lesions (Table 5).

**Comparison in the mRNA expression of LIMD1 and RB1**

Quantitative RT-PCR analysis revealed high reduction of LIMD1 mRNA expression (25.1 ± 19.04) than RB1 (3.8 ± 8.09) (Fig 4). Reduced mRNA expression of LIMD1 showed significant correlation with the gene’s methylation (see Additional file 6: Table S4). Half of these tumors (12/24) showed mean fold reduction in LIMD1 expression compare to 21% (5/24) in RB1. In Hep2 and UPCI: SCC084 cells RB1 expression was comparable to the normal tissues than that of reduced LIMD1 expression.

**Immuno-staining of pRB**

In normal epithelium, immuno-staining revealed intense nuclear and cytoplasmic pRB expression at basal layer, followed by gradual low cytoplasmic expression in differentiated cells (Fig 5). In dysplastic lesions as well as in HNSCC, both nuclear and cytoplasmic expression of pRB was observed. Low/medium level of pRB expression in...
Table 2 Summary of *LIMD1* mutations

| TNM stage | Nucleotide change | Exon1 | Exon5 | Mutations |
|-----------|------------------|-------|-------|-----------|
|           |                  | 470C → T | 660A del | 882C → T | 967T del | 1003C → A | 1068T → C | C del at intron4/exon5 |
|           | Amino acid change | Ser157 Phe | Frame shift | Thr294Thr | Frame shift | Pro335Thr | Gly356Gly | Splice-junction |
| Samples showing mutations |
| Moderate | L66 | - | - | - | - | - | - | - |
| Moderate | L139 | - | - | SNP³ | - | - | + | - |
| Severe | L144 | - | - | SNP³ | - | - | - | - |
| Severe | L126 | - | - | SNP³ | - | - | + | - |
| Severe | L135 | - | - | SNP³ | - | - | - | - |
| StageI | #292 | - | - | SNP³ | - | - | - | - |
| StageII | #2642 | - | - | - | - | - | + | - |
| StageI | #2772 | - | - | + | - | - | - | - |
| StageII | #7216 | - | - | - | + | - | - | - |
| StageII | #2073 | + | - | SNP³ | - | - | + | - |
| StageII | #5303 | - | + | - | - | - | - | - |
| StageII | #1234 | - | - | SNP³ | - | - | SNP³ | - |
| StageII | #2323 | - | - | + | - | - | - | - |
| StageII | #1108 | - | - | - | - | - | + | - |
| StageII | #7077 | + | - | - | - | - | - | - |
| StageII | #821 | - | - | - | - | - | + | - |
| StageII | #1367 | + | - | - | - | - | - | - |
| StageII | #1552 | - | - | SNP³ | - | + | - | - |
| StageII | #615 | - | - | - | + | - | - | - |
| StageII | #5090B | - | - | + | - | - | - | - |
| StageII | #816 | - | - | + | - | - | - | - |
| StageII | #526 | - | + | SNP³ | - | - | - | - |
| StageII | #1068 | - | - | SNP³ | - | + | - | - |
| StageII | #3893 | - | - | - | + | - | - | - |
| StageII | #308 | - | + | SNP³ | - | - | - | - |
| StageII | #6433 | - | + | SNP³ | - | - | - | - |
| StageII | #1332 | + | - | SNP³ | - | - | + | - |
| StageII | #2398 | - | - | - | + | - | - | - |
| StageII | #7059 | - | + | - | - | - | - | - |
| StageII | #1087 | - | - | + | - | - | - | - |
| StageII | #4075 | - | - | - | - | SNP³ | - | - |
| StageII | #2508 | - | + | - | - | + | - | - |
| StageII | #5114 | - | - | + | - | - | - | - |
| StageII | #5219 | - | + | - | - | - | - | - |
| StageII | #1084 | + | - | - | - | SNPa | - | - |
| StageII | #5184 | - | + | - | - | - | - | - |
| StageII | #944 | - | - | - | - | + | - | - |
| StageII | #2785 | - | - | SNP³ | - | - | - | + |
| StageII | #1774 | - | - | - | - | + | - | - |
| Cell lines | Hep2 | - | - | SNP³ | - | SNP³ | - | - |
| Cell lines | SCC084 | - | - | SNP³ | - | SNP³ | - | - |

*SNPa: rs267237; SNP³: rs267236; del: deletion; L: Dysplastic lesions; #: Invasive samples; ‘+’: Presence; ‘-’: Absence.*

Mutation frequency in HNSCC 6% (5/85) 9% (8/85) 7% (6/85) 4% (4/85) 2% (2/85) 9% (8/85) 4% (3/85)
was observed in 30% (6/20) of head and neck lesions contrary to frequent \textit{LIMD1} alterations (Table 6). In HPV18 positive Hep2 cells, pRB expression was mainly in cytosol, whereas in UPCI: SCC084 (HPV negative), it was expressed both in nucleus and cytoplasm. Concordance was seen between \textit{RB1} deletion with its RNA and protein expression (Fig 5).

Clinical and prognostic implications of \textit{LIMD1} and \textit{RB1} alterations

Using L1 primer, HPV DNA was detected in 56% (82/147) of head and neck lesions of which 84% (69/82) were HPV-16 positive, 11% (9/82) were HPV18 positive and the rest 5% (4/82) were positive for both HPV16/18. Univariate analysis showed significant association of \textit{RB1}...
deletion with HPV negativity ($P = 0.02$ for dysplastic lesions; $P = 0.01$ for HNSCC).

Log-rank test revealed significant poor survival in patients having alterations in at least one of these genes (Fig 6). Patients with both LIMD1 and RB1 alterations showed worse prognosis and absence of RB1 alterations did not change patients’ survival considerably (Fig 6). Multivariate analysis showed that LIMD1 molecular alterations ($P = 0.03$; HR, 4.5; CI, 1.1-17.8) along with tobacco addiction ($P = 0.05$; HR, 4.5; CI, 0.96-20.8) in absence of HPV ($P = 0.05$; HR, 0.28; CI, 0.08-0.99) were significant predictor for poor survival of patients with oral cavity cancer (Table 7). Moreover, patients with advanced grade tumor ($P = 0.50$; HR, 1.49; CI, 0.46-4.89) having nodal metastasis ($P = 0.42$; HR, 1.63; CI, 0.49-5.34) along with RB1 deletion ($P = 0.15$; HR, 2.27; CI, 0.74-7.02) showed a trend to have poorer survival, albeit being statistically insignificant.

### Discussion

Differential alterations of LIMD1 and RB1 during HNSCC development

To understand the role of LIMD1 and RB1 in HNSCC development, alterations of these genes were analyzed in 25 dysplastic lesions of head and neck, 58 HNSCC samples and two HNSCC cell lines. In dysplastic lesions LIMD1 mutations was less frequent than deletion and methylation (see Additional file 4: Table S2 and Additional file 5: Table S3). However, during progression of the tumor comparable frequencies of these alterations were seen, suggesting the deletion and methylation of LIMD1 as early events in this tumorigenesis. About 80% (67/83) of the samples showed any two of the alterations

### Table 3 Association study of LIMD1 different molecular alterations

|          | Dysplastic lesions | HNSCC |
|----------|--------------------|-------|
|          | Del+               | Del-  | Del+   | Del-   |
| Meth+    | 11                 | 8     | 21     | 15     |
| Meth-    | 4                  | 2     | 14     | 8      |
| Mut+     | 1                  | 1     | 18     | 13     |
| Mut-     | 14                 | 9     | 17     | 10     |
| Meth+    | 1                  | 1     | 15     | 21     |
| Meth-    | 1                  | 5     | 16     | 6      |

Del: Deletion; Meth: Methylation; Mut: Mutation; ‘+’: Positive; ‘-’: Negative.

### Table 4 Allele frequency of (CA)n polymorphism in the LIMD1 gene in head and neck cancer cases and control

| Allele (CA)n repeats | No. of alleles | No. of (CA)n repeats | No. of alleles | P* |
|----------------------|----------------|----------------------|----------------|----|
| (CA)9                | 9              | 7                    | 238            | 0.6571 |
| (CA)13               | 13             | 4                    | 136            | 0.00012 |
| (CA)17               | 17             | 23                   | 782            | 0.0592 |
| (CA)19               | 19             | 97                   | 32.99          | 0.00139 |
| (CA)20               | 20             | 66                   | 22.45          | 0.000005 |
| (CA)21               | 21             | 21                   | 7.14           | 0.075134 |
| (CA)24               | 24             | 30                   | 10.2           | 0.05427 |
| (CA)26               | 26             | 2                    | 0.68           | 0.0067 |
| (CA)27               | 27             | 19                   | 6.46           | 0.0728 |
| (CA)30               | 30             | 10                   | 3.4            | 0.1316 |
| (CA)32               | 32             | 14                   | 4.76           | 0.0082 |
| (CA)38               | 38             | 1                    | 0.34           | 0.259 |

Total no. of alleles 294 100 374 100

| Genotype | Case | Control | P* |
|----------|------|---------|----|
| (CA)9/(CA)12 | 6    | 11      | 15.94 | 0.2434 |
| (CA)12/(CA)10 | 33   | 56      | 81.16 | 0.0002 |
| (CA)12/(CA)20 | 19   | 2       | 2.89  | 0.00003 |
| (CA)20/(CA)21 | 5    | 0       | 0.0247 |
| (CA)32/(CA)34 | 1    | 0       | 0.4851 |
| (CA)34/(CA)32 | 1    | 0       | 0.4851 |

# From Fisher’s exact test.

OR, Odds ratio

CI, Confidence interval

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in **LIMD1** supporting the modified Knudson two hit hypothesis as a candidate TSG. High frequency (94%, 78/83) of **LIMD1** alterations (deletion/methylation/mutation) than **RB1** was seen in the samples. Majority of mutations in **LIMD1** were present in and around the pRB binding domain of exon1. Unlike us, Huggins et al. detected very low frequency (4%, 6/165) of mutation in exon1 of **LIMD1** at different positions in breast carcinoma [21]. In our samples, about 42% (14/33) of the mutations produced non-functional truncated **LIMD1**

**Table 5 Association study of **LIMD1** alterations with **RB1** deletion**

|           | Stage I+II | Stage III+IV |
|-----------|------------|--------------|
| **LIMD1** |            |              |
| **RB1**   |            |              |
| Del+      | 2          | 13           |
| Del-      | 21         | 24           |
| ALT+      | 0          | 0            |
| ALT-      | 2          | 2            |
| **P**     | 0.6637     | 0.4326       |

Alt: Overall molecular alterations; Del: Deletion; ‘+’: Positive; ‘-’: Negative.

**Figure 4 (A).** Quantitative RT-PCR analysis showing **LIMD1** and **RB1** expression in HNSCC samples (n = 24), Hep2 and UPCI: SCC084 cell lines. Bars represented the gene expression normalized to β2-microglobulin and relative to normal counterpart. Black bar represented **RB** expression and cross line filled bar represented **LIMD1** expression.

**Figure 5** Immunohistochemical staining patterns of pRB in dysplastic lesions (L), HNSCC samples (T), normal tissues (Normal) and HNSCC cell lines (Hep2, UPCI: SCC084) samples showing high (a), low/negative (b) expression of pRB protein (indicated by arrows). In #797T, **RB1** was not deleted whereas in #944T, this gene was deleted. The regions marked within the circle (20x) were magnified to 40x at inset.
proteins due to premature termination or splice-junction mutations (Table 2). The significance of synonymous mutations at two SNPs was not clear.

**RB1** deletion was infrequent with much lower frequency in dysplastic lesions than invasive samples, suggesting it less likely to be a candidate TSG in this tumor. Similar to other reports [22], there was a strong association between **RB1** deletion and reduced pRB expression in our samples \((P = 0.0006)\) (Table 6), indicating deletion as the main cause of **RB1** inactivation. The differential downregulation in mRNA expression of **LIMD1** and **RB1** was concordant with their molecular alterations, reflecting genetic imbalances transmitted in transcript level. Intense pRB staining in basal layer of normal epithelium of oral cavity compare to the differentiated cells indicates differential regulation of pRB during differentiation. Similar reports were also found in normal epithelium of esophagus and cervix [23-25]. **LIMD1** staining has not been done due to unavailability of commercial antibody. In dysplastic lesions and in HNSCC, pRB expression was seen both in nucleus and cytoplasm where **RB1** deletion was absent, similar to basal layer of normal epithelium. However the functional status of pRB in tumor cells is not clear. The reduced level of **LIMD1** in tumor cells might destabilize the pRB-E2F interaction and chromatin remodeling complex, resulting deregulation of cell cycle.

**Identification of susceptible allele of **LIMD1**

As **LIMD1** has been suggested to be a candidate TSG in HNSCC, our next attempt was to search for a susceptible allele of this gene, if any, associated with HNSCC risk. The case-control study identified (CA)20 as the risk allele both in its homozygous and heterozygous state for HNSCC development. Similar repeat length polymorphism in upstream of several genes has been reported [26-28]. The significance of the (CA)19 repeat variations at upstream of **LIMD1** was not clear. Regarding the conformation, the (CA)19 repeat length might be a critical point. Though the significance of critical (CA)20

| Sample No.; HPV status | Genetic alterations | mRNA status | Protein expression | Genetic/epigenetic alterations | mRNA status |
|------------------------|--------------------|-------------|--------------------|-------------------------------|-------------|
| L127; HPV-             | D-                 | ND          | Normal             | D+Me+Mu-                      | ND          |
| L139; HPV-             | D-                 | ND          | Normal             | D+Me+Mu-                      | ND          |
| L154; HPV-             | D-                 | ND          | Normal             | D+Me+Mu-                      | ND          |
| L162; HPV-             | D-                 | ND          | Normal             | D+Me+Mu-                      | ND          |
| L158; HPV16            | D-                 | ND          | Medium             | D+Me+Mu-                      | ND          |
| HNSCC samples          |                    |             |                    |                               |             |
| #1004; HPV-            | D-                 | Normal      | Normal             | D+Me+Mu-                      | ND          |
| #5165; HPV-            | D-                 | Normal      | Normal             | D+Me+Mu-                      | ND          |
| #6817;HPV16            | D+                 | ↓           | Medium             | D+Me+Mu-                      | ND          |
| #2884; HPV-            | D-                 | Normal      | Normal             | D-Me+Mu-                      | ND          |
| #1108; HPV-            | D+                 | ↓           | Medium             | D-Me+Mu+                      | ND          |
| #1774;HPV16            | D+                 | ↓           | Low                | D-Me+Mu+                      | ND          |
| #872; HPV-             | D-                 | Normal      | Normal             | D-Me-Mu-                      | ND          |
| #5303; HPV-            | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |
| #6907;HPV16            | D-                 | ↓           | Low                | D-Me+Mu+                      | ND          |
| #1087; HPV-            | D-                 | Normal      | Normal             | D-Me-Mu-                      | ND          |
| #944; HPV-             | D+                 | ↓           | Low                | D-Me+Mu+                      | ND          |
| #5733;HPV16            | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |
| #6392; HPV-            | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |
| #7077; HPV-            | D-                 | Normal      | Normal             | D-Me-Mu+                      | ND          |
| #797; HPV-             | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |
| Hep2, HPV18            | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |
| UPCI:SCC084, HPV-      | D-                 | Normal      | Normal             | D-Me+Mu+                      | ND          |

L: Dysplastic lesions; # HNSCC samples; D: Deletion; Me: Methylation; Mu: Mutation; ‘+’: Positive; ‘-’: Negative; ‘↓’: Down expression; ND: Not done.

Table 6 Correlation between mRNA and protein expression of **RB1** and their association with **LIMD1** molecular alterations.
repeat as risk for this tumor development is not fully understood, it seems that this repeat length might destabilize Z-DNA conformation, causing transcriptional repression of LIMD1. Similar effect might also be imposed by (CA)$_{21}$-(CA)$_{38}$ alleles, but due to their low prevalence in our study population, statistical significance has not been observed. In light of the strength of associations and the increased risk for HNSCC, further research is warranted to explore the potential underlying mechanism(s) involved.

Table 7 Multivariate analysis of overall survival of oral cavity cancer patients with different clinicopathological parameters.

| Variable          | Overall survival | P-value | Hazard ratio (HR) | 95% CI for HR |
|-------------------|------------------|---------|------------------|---------------|
| LIMD1 alteration  | 0.0318           | 4.51    | 1.14-17.82       |               |
| RB deletion       | 0.1535           | 2.27    | 0.74-7.02        |               |
| Stage             | 0.5144           | 0.79    | 0.39-1.59        |               |
| Grade             | 0.5044           | 1.49    | 0.46-4.89        |               |
| Age               | 0.7786           | 0.84    | 0.25-2.85        |               |
| Node              | 0.4228           | 1.63    | 0.49-5.34        |               |
| HPV               | 0.0498           | 0.28    | 0.08-0.99        |               |
| Tobacco           | 0.0559           | 4.49    | 0.96-20.89       |               |
| Alcohol           | 0.2659           | 0.29    | 0.03-2.52        |               |

CI, Confidence interval.
Bold letters represent significance level.

Association of LIMD1 and RB1 alterations with HNSCC progression and prognosis

Unlike methylation and deletion, significant association of LIMD1 mutation with tumor progression indicates that mutation might have some additive effect in inactivation of this gene. In multivariate analysis, association of LIMD1 alterations along with tobacco addictions HPV negativity and poor patients' outcome suggests LIMD1 as predictive clinical marker in progression of HNSCC. Agreed with similar findings [29-31], significant association was seen in RB1 deletion with HPV negative samples and with HNSCC progression. Likewise, a trend towards significance of RB1 deletion in advance grade tumors, with nodes of pathology has also been observed [31]. However, worse prognosis of the patients having both LIMD1 and RB1 alterations suggests RB1 inactivation might have some synergistic impact in HNSCC development. Comparatively better survival of patients with LIMD1+RB1- (LIMD1 unaltered & RB1 deleted) than LIMD1-RB1+ (LIMD1 altered & RB1 unaltered), indicates LIMD1 as a key regulator of the disease.

Conclusion

Thus, it can be concluded that LIMD1 is a susceptible gene for HNSCC development and its alterations, alone or with RB1 alterations acts as an important prognostic marker in this cancer.
Additional file 1: Oligonucleotide primers used in the study. The data report the primers used in the study. Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-9-58-S1.DOCX]

Additional file 2: A representative chromatograph. The chromatogram represents C/T heterozygous in PBL at SNP rs26737 and C → T mutation in Tumor sample. Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-9-58-S2.TIFF]

Additional file 3: Legend for the Figure S1. Short explanation for the Figure S1. Click here for file
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Additional file 4: LIMD1 overall molecular alterations including deletion, methylation, mutation and mRNA expression. The data provided represent the overall molecular alterations ie deletion, promoter methylation, mutation of LIMD1 along with the mRNA expression in each of individual sample studied. Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-9-58-S4.XLS]

Additional file 5: Pattern of LIMD1 alterations in HNSCC during progression of the disease. The data provided represent the frequencies of deletion, methylation and mutation during the progression of HNSCC. Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-9-58-S5.DOCX]

Additional file 6: Correlation between LIMD1 methylation and mRNA expression. The data provided represent the correlation between LIMD1 promoter methylation and its mRNA expression in each individual sample. Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-9-58-S6.DOCX]

Abbreviations
HNSCC: Head and neck squamous cell carcinoma.

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The authors declare that they have no competing interests.

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