Cancer Genetics 244 (2020) 21–29

Contents lists available at ScienceDirect

Cancer Genetics

journal homepage: www.elsevier.com/locate/cancergen

LINE-1 retrotransposon encoded ORF1p expression and promoter methylation in oral squamous cell carcinoma: a pilot study

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ARTICLE INFO

Article history:
Received 19 July 2019
Revised 24 January 2020
Accepted 27 January 2020

Keywords:
LINE1 retrotransposon
LINE1 promoter
LINE1 ORF1p antibody
LINE1 retrotransposon
Oral cancer
OSCC

ABSTRACT

Oral squamous cell carcinoma (OSCC) is highly predominant in India due to excessive use of tobacco. Here we investigated Long INterpersed Element 1 (LINE or L1) retrotransposon activity in OSCC samples in the same population. There are almost 500,000 copies of L1 occupied around 30% of the human genome. Although most of them are inactive, around 150–200 copies are actively jumping in a human genome. L1 encodes two proteins designated as ORF1p and ORF2p and expression of both proteins are critical for the process of retrotransposition. Here we have analyzed L1 ORF1p expression in a small cohort ($n = 15$) of paired cancer-normal tissues obtained from operated oral cancer patients. Immunohistochemistry (IHC) with the human ORF1 antibody showed the presence of ORF1p in almost 60% cancer samples, and few of them also showed aberrant p53 expression. Investigating L1 promoter methylation status, showed certain trends towards hypomethylation of the L1 promoter in cancer tissues compared to its normal counterpart. Our data raise the possibility that L1ORF1p expression might have some role in the onset and progression of this particular type of cancer.

Introduction

Retrotransposons are mobile genetic elements that move within a genome using ribonucleic acid (RNA) as an intermediate to produce new insertions, which can disrupt genes and expand genomes due to increases in copy number [1,2]. Long INterperserd Element 1 (LINE-1 or L1) is the only autonomously active retrotransposon in the human genome, with around five hundred thousand copies occupying around 17% of the human genome [1–5]. Although; extremely abundant, only a subset of L1s (~80–100) is retrotransposition competent L1s (RC-L1s), actively retrotransposing in any given human [6]. A RC-L1 is 6 kb in length and encodes two proteins (ORF1p and ORF2p). Both proteins are required for the process of retrotransposition [7]. ORF1p encodes a protein with RNA binding and nucleic acid chaperone activities [8], whereas ORF2 encodes a protein with reverse transcriptase (RT) [9] and endonuclease (EN) [10] activities.

Due to their potential to function as insertional mutagens, L1s are generally silenced in somatic cells through epigenetic and post-transcriptional mechanism such as heterochromatinization of L1 sequences, CpG methylation in the L1 promoter, premature termination of L1 transcript, small RNA induced silencing, host cellular factor-mediated retrotransposition inhibition and others [11–14]. The L1 5′-UTR contains 39 CpG sites, which when methylated, are associated with repression of L1 RNA expression [14,15]. However, recent transgenic animal models and deep sequencing studies revealed that a subset of L1s escaped repression and resulting in high expression of these elements in germ cells, early stages of development, certain regions of brain, and cancers [16-22].

Oral squamous cell carcinoma (OSCC) that arises from epithelial cells represents 95% of all forms of head and neck cancer [23,24].

List Of Abbreviation Used In Manuscript: OSCC, Oral Squamous Cell Carcinoma; LINE-1, Long INterpersed Element-1; ORF, Open Reading Frame; IHC, Immunohistochemistry; RRM, RNA Recognition Motif; RNA, Ribonucleic Acid; DNA, Deoxyribonucleic Acid; kDa, kilo Dalton; UTR, Untranslated region; TP53, Tumor Protein 53; PPs, processed pseudogenes; X-gal, 5–bromo–4–chloro–3-indolyl-β-D-galactopyranoside; IPTG, Isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; NaCl, sodium chloride; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; ECL, Enhanced chemiluminescence; HRP, Horseradish peroxidase; x g, times gravity; MCF-7, Michigan Cancer Foundation-7; PBS, Phosphate Buffered Saline; BSA, Bovine Serum Albumin; FFPE, Formalin-Fixed Paraffin Embedded; EDTA, Ethylene Diamine Tetra-Acetic Acid; TBST, TrisBuffered Saline-Tween; DAB, 3-3′ Diaaminobenzidinetetrahydrochloride (DAB substrate).

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https://doi.org/10.1016/j.cancergen.2020.01.050
2210-7762/

It is the eighth most common cancer in the world, with a very high mortality rate. The five-year survival rate of patients after surgery is around 50% [23,24]**.** The major risk factors for OSCC include tobacco use, alcohol consumption and infection with human papillomavirus (HPV) [25–27]. Similar to all other types of cancer, OSCC is thought to be initiated and progress by a series of genetic alterations [28–30]. Whole-genome and exome sequencing revealed that several genes, like tumor protein 53 (*TP53)* and *Notch,* are frequently mutated in OSCC [28,29]. Aberrant deoxyribonucleic acid (DNA) methylations, particularly at the promoter regions, are common to all types of cancers. Numerous studies have reported that hypermethylation of tumor suppressor promoters in cancer leads to transcriptional silencing of these genes [31–34]. In addition to, gene specific hypermethylation, global hypomethylation is observed during transformation [31]. Notably, retrotransposons are often hypomethylated in cancer resulting in the expression of retrotransposon-encoded proteins [21,22,35–37].

While whole-genome sequencing (WGS) of tumor samples has uncovered high rates of L1 retrotransposition in certain types of cancer, particularly those of epithelial cell origin, the frequency of new L1 insertions differs across cancers sampled to date (colorectal, prostate, ovarian, multiple myeloma and glioblastoma) [38,39]. Strikingly, in some selected cancers (e.g., colorectal cancer) the number of somatic L1 insertion frequency is striking with more than 100 retrotransposition events detected in one tumor [38]. Although our understanding of L1 activity in cancers has increased dramatically over the past five years, significant gaps remain in our knowledge, including the spectrum of cancers displaying retrotransposon expression.

In this study, we investigated L1ORF1p expression and L1 promoter methylation in 15 paired cancer normal tissues obtained from oral cancer patients. We observed increased hypomethylation of L1- promoter in cancer tissues compared to matched normal. In addition, by immunohistochemistry (IHC) analysis, we detected seven out of twelve samples showed a detectable level of L1 ORF1p expression, many of which also display aberrant p53 levels. Collectively, these data highlight the L1 retrotransposon as a novel factor with the potential to contribute to the onset and progression of OSCC.

Materials and methods

Specimen collection of tissue specimens

All the paired normal cancer tissues were collected postoperatively following proper consent from the patient and their immediate family member from the Acharya Tulsi Regional Cancer Treatment and Research Institute, Bikaner, Rajasthan, India. The details of patients used in this study are present supplementary Table 1. Following initial collection, samples were stored in RNA later solution (Qiagen) at −20 °C. Subsequently, these tissues were used for genomic DNA and protein isolation, along with the production of formalin-fixed paraffin-embedded blocks. All investigations were conducted in accordance with ethical principles embodied in the declaration of tissue request and material transfer agreement (IHEC No. BT/IHEC-IITR/2017/6673; Institute Human Ethics Committee (IHEC), Indian Institute of Technology Roorkee, Uttarakhand, India).

Genomic DNA extraction

~100 mg tissue was washed with phosphate buffer saline (PBS) followed by homogenization of the sample in liquid nitrogen using a mortar and pestle. From homogenized tissue, genomic DNA was extracted using the Blood &Tissue DNeasy mini kit (Qiagen) per manufacturer instruction; DNA was eluted in 100 µl TE buffer. The integrity of the DNA was checked in a 0.6% agarose gel.

LINE-1 promoter methylation analysis

Bisulfite conversion of genomic DNA (1 µg) isolated from tissue specimens was performed using the Epitect kit (Qiagen) following manufacturer's instructions. A 363 bp sequence within the L1 5′UTR region (nucleotide number 209–572; L1HS, RepBase) [15], which contains twenty CpG dinucleotides, was amplified using methylated primer set (For: 5′-AAGGGGTTAGGGAGTTTTTTT-3′ and Rev: 5′- TATCTATACCCTACCCCCAAAA-3′). Briefly, the 50 µl PCR reaction was set up using 2X GoTaq green (Promega) and 200 ng template bisulfite treated DNA. Untreated genomic DNA was used as control. No PCR amplification with the untreated DNA template suggested that the genomic DNA was 100% converted by bisulfite treatment. PCR conditions as follows: one cycle at 94 °C for 30 s followed by 30 cycles at 94 °C for 20 s, 54 °C for 30 s and 72 °C for 60 s and finally one cycle at 72 °C for 5 min. The PCR products were resolved in a 1.2% agarose gel. Bands were excised, gel extracted and subcloned in the pGEM-T vector (promega) followed by transformation and blue-white screening. Plasmid DNA was extracted from positive colonies (white) using a mini-prep DNA kit (Qiagen). Clones were first checked by electrophoresis in 1.2% agarose gel, and five clones from each sample sent for Sanger sequencing using T7 promoter and SP6 universal primers. The sequenced clones were first characterized using Repeat masker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker), which allowed for subfamily annotation. Most of the sequences belonged to either the L1-HS or $L1_{P1}$ subfamily. The sequence was manually inspected to see whether C is intact (signature of methylated C) or changed (marked as unmethylated C) in the CpGs [40,41]. We used quantification tool for methylation analysis (QUMA) and BioEdit programs (freely available software) to analyze the bisulfite sequence data for methylation analysis [42,43]

Cell culture

HEK293T (human embryonic kidney) cells were maintained in a CO_2 incubator at 37 °C and 5% CO_2concentration in high glucose Dulbecco's modified Eagle medium (DMEM) with *L*– glutamine (Gibco) supplemented with 10% fetal bovine Calf serum and 100 U/ml penicillin-streptomycin. Cells were maintained for a few passages and frozen as aliquots; for experiments, cells were subcultured no more than a month to ensure a low number of population doubling.

Protein extraction and immunoblotting

Whole-cell lysate was prepared from MCF-7 and HeLa cells using lysis buffer A [composition: 20 mM Tris-Cl pH 7.8,137 mM sodium chloride (NaCl) and 1% NP-40 supplemented with 1X protease inhibitor cocktail (Roche)]. The lysate was centrifuged at 2500 × *g* (times gravity) for 5 min at 4 °C, and the supernatant was transferred to a new 1.5 mL tube, which was stored at −70 °C until further use. For the preparation of cancer and normal tissue lysate, around 150–200 mg of frozen tissue was placed in liquid nitrogen and crushed using a mortar and pestle. Next, the sample was transferred to a 1.5 ml tube containing 250 µl of cold RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate,0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-Cl pH-8.0 with protease inhibitor cocktail (Roche)]. The crushed tissue was then passed through an 18-gage needle 5–8 times, followed by incubation on ice for 45 min with intermittent mixing. Finally, the lysate was centrifuged at 12,000 × *g* for 10 min at 4 °C; the supernatant

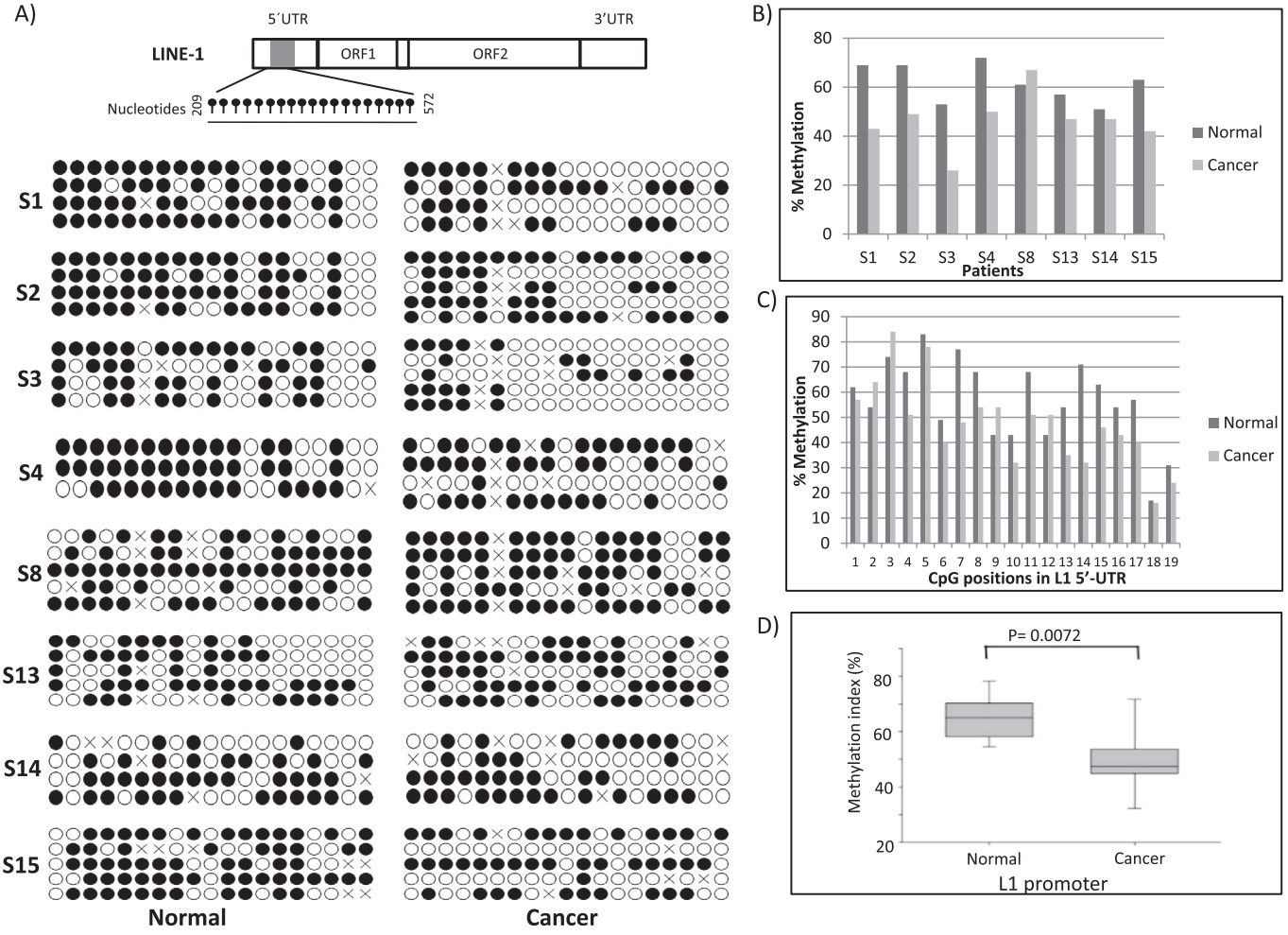

Fig. 1. Methylation analysis of the human L1 promoter in matched OSCC and normal tissues. (A) Scheme of a full-length active human L1 containing a 5′-UTR encoding an internal promoter, ORF1p and ORF2p, and a 3′-UTR. The positions of 19 CpG residues analyzed in this study are shown as lollipops. Bisulfite analysis of eight paired normal cancer tissues to determine DNA methylation levels of the L1 promoter in OSCC tissues. The quantification tool for methylation analysis (QUMA) software was used to analyze bisulfite sequence data [42]. An average of 5 clones were Sanger sequenced for each patient sample. The position of each CpG residue is relative to the sequence of L1-Hs (Repbase) [15]. Open and closed circles denote unmethylated and methylated cytosines, respectively, in the CpG dinucleotides. Cross indicates the mutated CpG site. (**B)**% Methylation of 8 OSCC patient tissues relative to matched samples. (**C)** Methylation levels for each of the 19 CpG dinucleotides in the L1 promoter assayed across the matched tissues. (**D)** Methylation index for the L1 promoter of OSCC compared to matched normal tissue. A two-tailed paired *t*-test calculated the p-value.

was transferred to a new tube and stored at −70 °C until further use. The Bradford reagent (Bio-Rad) was used to estimate the protein concentration. Protein lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini protein Tetra cell (Bio-Rad)) and wet transferred by applying 100 V for 75 min (Bio-Rad mini trans blot electrophoretic transfer cell) to nitrocellulose membrane (Millipore). Protein was detected using the following primary antibody. Polyclonal rabbit human α-L1 ORF1p (RRM) (1:33,000) [44], α- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:6000) (Santa Cruz Biotechnology), α-FLAG (1:3000) (Sigma), α-p53 (DO7) Mouse Monoclonal antibody (Sigma-Aldrich). Secondary α-rabbit HRP and secondary α-mouse HRP were purchased from Jacksons Immuno Research Laboratories, USA. Western blots were developed using enhanced chemiluminescence (ECL) western blotting detection reagent (Pierce) as per the manufacturer's instructions. The bands were detected by exposing the blot on X-ray film (Hyper film from GE Healthcare.

Immunohistochemistry (IHC)

Paraffin-embedded normal and cancer tissue sections on glass slides were deparaffinized, rehydrated in descending grade of ethanol solutions before proceeding for antigen retrieval. The antigen retrieval step was adapted from "abcam protocol" available at (http://www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol). Briefly, antigen retrieval was performed in a common household vegetable steamer (pressure cooker) using Tris-EDTA (ethylene diamine terta acetic acid) antigen retrieval buffer (10 mMTris base, 1 mM EDTA solution, 0.05% Tween 20, pH-9.0), then the slides were washed 2 × 5 min each in tris buffered saline tween (TBST) (1X TBS containing 0.025% Triton-X100) and then blocked in blocking solution [1% BSA (bovine serum albumin) in 1XTBST] for 1 h at room temperature. After that, slides were incubated with polyclonal rabbit α-ORF1p (RRM) antibody (1:500 diluted in blocking reagent) at 4 °C overnight in a humid chamber. The next day, slides were washed with 1XTBST and treated with 0.3% hydrogen peroxide to quench any peroxidise present within the tissue. Slides were then incubated with secondary antibody ((1:500 dilution goat α-rabbit horseradish peroxidise (HRP) (Jacksons Immuno Research)) for an hour at room temperature. The slides were washed again 3 × 10 min at room temperature with gentle agitation. Signals were visualized by adding 3–3÷ Diaaminobenzidinetetrahydrochloride (DAB substrate) solution to the slides and counterstained

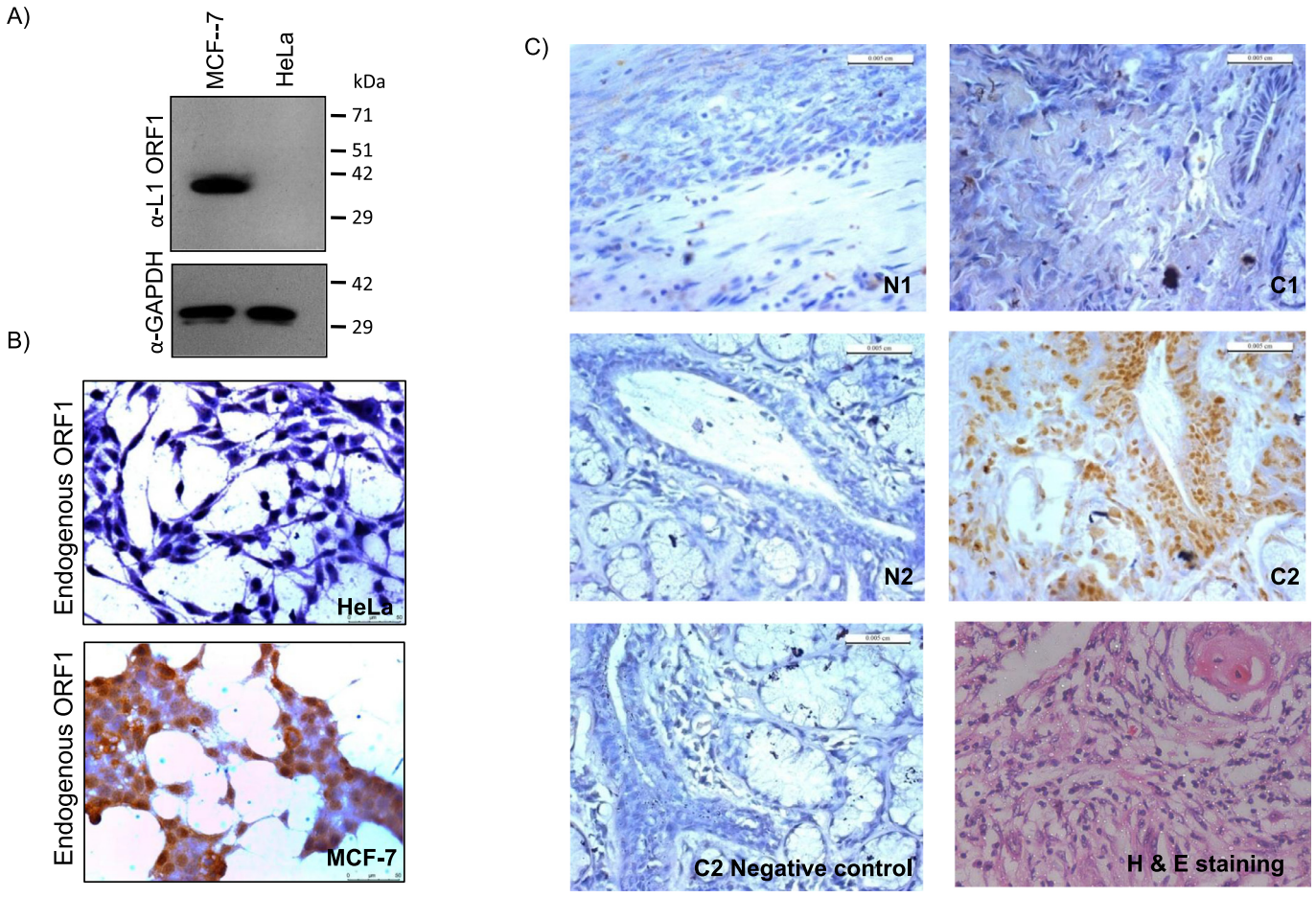

Fig. 2. Human L1 ORF1 protein expression in operated oral cancer samples. (A) Validation of human α-L1 ORF1p (RRM) antibody by western blotting in untransfected HeLa and MCF-7 cancer cell lines. Panel 1 showsen dogenous L1ORF1p in HeLa and MCF-7 cells. Immunoblot with α-GAPDH serves as a loading control (panel 2). (B) Immunocytochemistry with anti-L1ORF1p (RRM) antibody in untransfected HeLa and MCF-7 cancer cells. **(C)** IHC staining of oral cancer and matched normal tissues using α-L1 ORF1p (RRM) antibody. All images were collected at 40X magnification. N–Normal; C–Cancer; the number indicates the patient number (e.g., C1- Patient 1 oral cancer tissue; N1- patient 1 matching normal tissue). The cancer tissue section from patient number 2 not treated with primary antibody served as a negative control (bottom left panel); Hematoxylin and eosin staining of sample C2 served as a positive control (bottom right panel).

with haematoxylin, (Himedia) dehydrated with ascending order of ethanol and mounted with DPX mounting media. Images were captured using a light microscope (Leica Microsystems) equipped with a camera. The intensity of DAB stained regions was measured with ImageRatio software [45] and plotted as the percentage of expression.

Statistical analysis

The methylation value was calculated as mC/(mC + hmC) for all examined CpGs for a particular patient where hmC = hypomethylated Cytosine and mC = methylated Cytosine. The hypomethylation index for the LINE-1 elements in paired tumor and normal tissues was calculated as a mean value of mC/(mC + hmC) for all examined CpG dinucleotides. The One-Sample Kolmogorov-Smirnov test was used to evaluate fitness to a normal distribution of continuous parameters. A Paired *t*-test was used to determine if there was a statistically significant change in the methylation status of LINE-1 in OSCC tumor versus paired normal (Supplementary text). All analyses were performed using the sigma plot 13 package (manufacturer or website. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Loss of DNA methylation at CpGs within the L1 5′-UTR in oral cancer samples

Epigenetic silencing of the L1 5′-UTR by DNA methylation is a common means to inactivate L1 expression and, ultimately, retrotransposition. Epigenetic alterations are frequent in cancers. Indeed, several studies have reported reduced methylation of the L1 promoter in a variety of cancers [21,22,35–37]. To date, the methylated state of the L1 5′-UTR in OSCC remained unexamined; therefore, we performed bisulfite conversion analysis of genomic DNA across nine paired normal-cancer tissues followed by PCR, subcloning of amplicons, and Sanger sequencing to ascertain the methylation level of the L1 promoter (Supplementary Figure 1). Specifically, we amplified a 363 bp region of the L1 promoter (nucleotide sequence 209–572, L1Hs from Repbase [15,40,41], which contains 19 CpG sites and the resultant amplicons were sequenced (Fig. 1A; Supplementary Figure 1A). For each sample pair (e.g., OSCC/matched normal), five independent clones were sequenced (Fig. 1A). Our sequence analysis uncovered that most of the L1 amplicons belonged to the major active L1 subfamily, L1-Hs (Supplementary text; Supplementary Table 2), suggesting that we were tracking potentially active L1s in the tumor tissues. The bisulfite

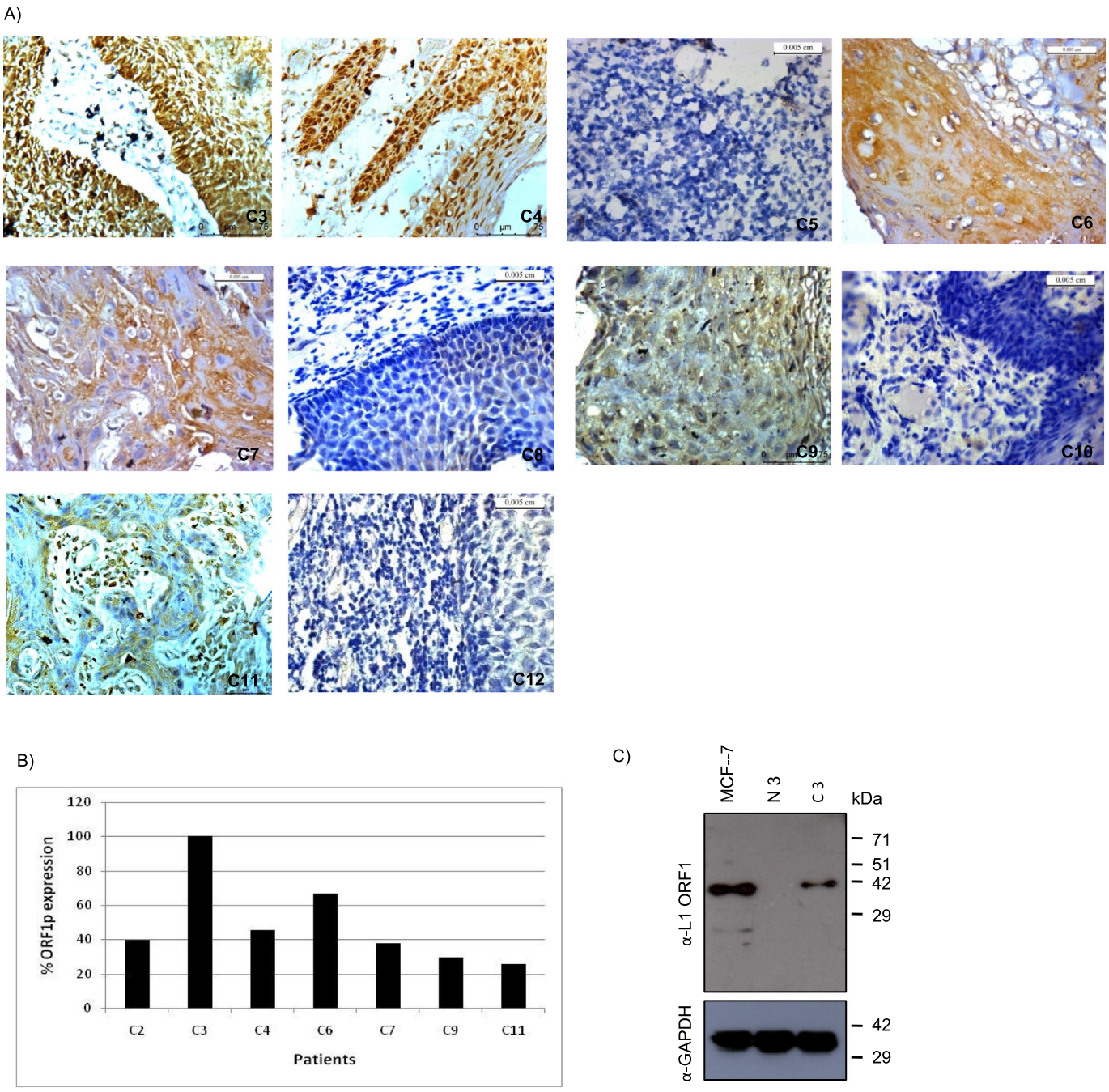

Fig. 3. ORF1p immunohistochemistry analysis across oral cancer samples using the human α-L1 ORF1p (RRM) antibody. (**A**) IHC was performed for ten more OSCC samples to detect ORF1p expression. All pictures were taken in 40X magnification. (**B**) Quantification of DAB signal intensities, a measure of ORF1p expression in different cancer samples. Values were calculated and plotted using ImmunoRatio software [44]. (**C**) Detection of L1ORF1p by western blot analysis in whole tissue lysate obtained from sample C3. The corresponding matched normal (N3, lane 2) served as a negative control, and the total lysate from the MCF-7 cell line (lane 1) was used as a positive control.

sequences were analyzed using the quantification tool for methylation analysis (QUMA) [42] and BioEdit programs [43].

Of the eight paired samples analyzed, reduced methylation of L1 5′UTR was detected in five of the tumor tissues (Sample S1, S2, S3, S4, and S15) relative to matched normal tissue (Fig. 1B). When comparing normal to matched OSCC tissue, S3 exhibited the largest difference in CpG methylation in the cancer tissue, whereas S8 and S14 displayed no detectable difference in methylation level between cancer and matched normal tissue (Fig. 1B).

Further interrogation of specific CpGs within the amplified L1 5′-UTR sequence indicates that hypomethylation across CpG does not occur uniformly. Specifically, some positions are more prone to loss methyl group (site: 4, 7, 8, 11, 13, 14, 15, 16 and 17), while several (site:1, 2, 3, 5, 6, 9, 18, and 19) show no significant differences in methylation state between the cancer and paired normal (Fig. 1C). Quantification confirmed distinct differences in L1 5′-UTR methylation when comparing normal (methylation index = 65 ± 2.8) to OSCC (44.5 ± 3.9) (Fig. 1D; Supplementary text). These data demonstrate reduced methylation of L1 pro-

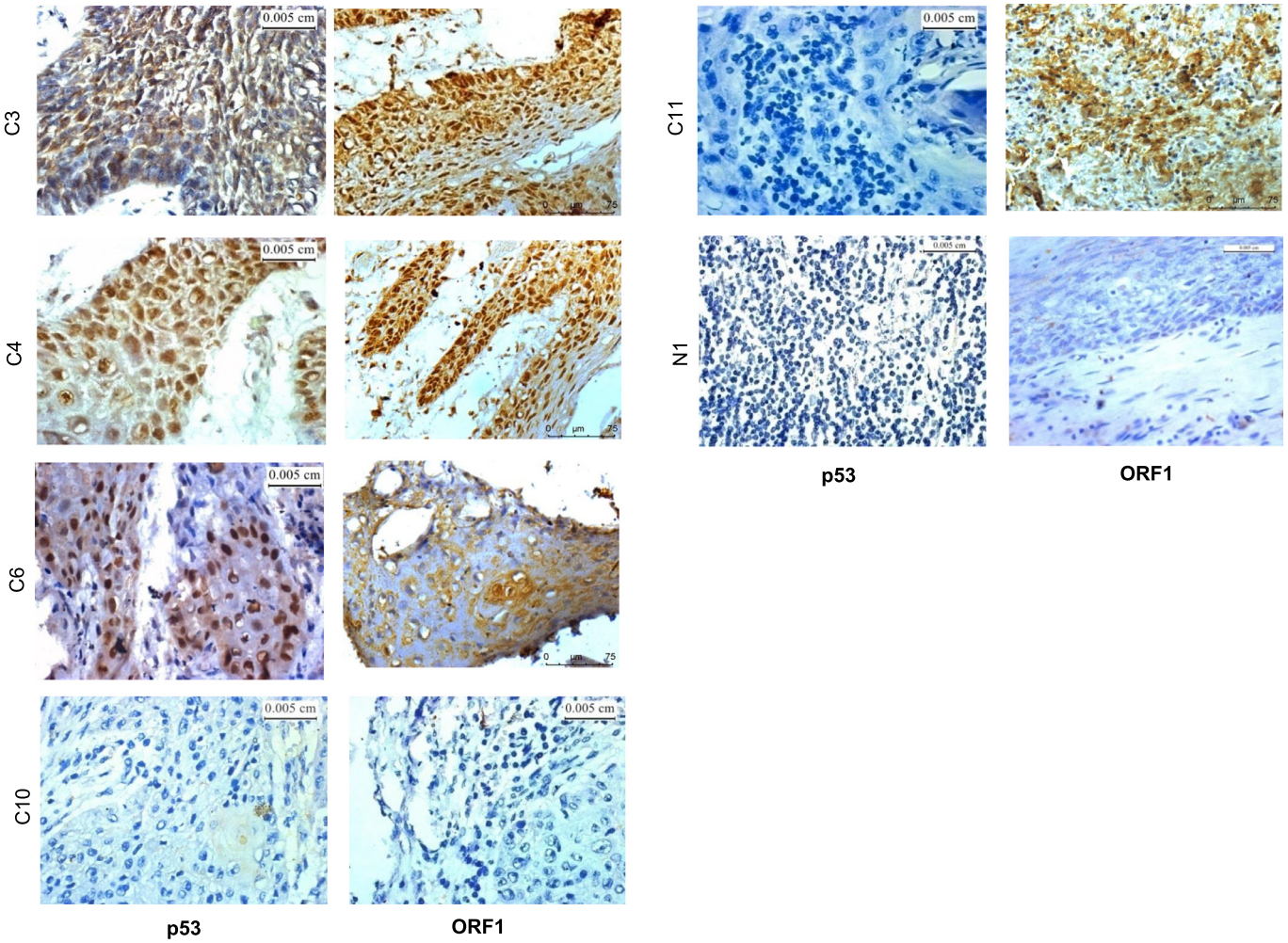

Fig. 4. L1 ORF1p expression in OSCC correlates with aberrant expression of p53. Five oral cancer samples (C3, C4, C6, C10, and C11) were tested for p53 expression by IHC. Among those five samples, four (C3, C4, C6, and C11) were ORF1p positive, and one (C10) was negative. Three (C3, C4, and C6) out of five samples showed significant p53 expression. Normal tissue from patient one (N1) served as a negative control for both ORF1p and p53 staining.

moter CpG dinucleotide in OSCC cancer tissues compared to paired normal.

To determine whether the loss of epigenetic silencing in the 5′-UTR is associated with L1 protein expression – a requirement for the production of new insertions, we assayed tissue samples using a polyclonal antibody specific to the RNA recognition motif (RRM) domain of ORF1p [45]. As a control, western blot analysis was carried out using lysates from MCF-7 (Michigan Cancer foundation-7), a breast cancer cell line known to express L1 and one not known to (HeLa, cervical cancer cell line) (Fig. 2A). Indeed, we observe a robust band at 40 kgdalton (kDa) – the predicted size of ORF1p – in MCF-7 cells but not in HeLa. Next, we performed immunocytochemistry on MCF-7 and HeLa cell lines using the same ORF1p antibody as an additional control. In agreement with our western blot data, we observe staining almost exclusively in MCF-7 cells (Fig. 2B). Similar to previous reports, we observe ORF1p primarily in the cytoplasm [46–51].

The five tissue samples displaying hypomethylated L1 5′-UTRs were further characterized by immunohistochemistry to assay, whether loss of epigenetic silencing is associated with increased L1 protein expression. Indeed, in three of these tissues (S2, S3, and S4) we observe significant staining using an antibody specific for the RRM domain of ORF1p (Fig. 2C and Fig. 3A), supporting a link between hypomethylation of the L1 5′-UTR and L1 protein expression in OSCC.

Detectable expression of L1 ORF1p in oral cancer samples

Next, we tested how common, L1 ORF1p expression is across OSCC samples. To this end, we carried out IHC on a total of twelve post-operated oral cancer samples. The neoplastic nature of all cancer samples used in this study was confirmed by hematoxylin and eosin staining (H&E staining). A representative of this staining is shown in Fig. 2C (right bottom panel). Using the anti-ORF1p antibody, we detected some level of expression in over half of our samples (7 out of 12; 58%)(Fig. 2C, Fig. 3). Supporting cancer-specific expression in OSCC, we did not observe any staining using anti-ORF1p in normal oral tissues tested (Fig. 2C). Incubation of a cancer tissue section from patient 2 (C2) without primary antibody functioned as a negative control (Fig. 2C, left bottom panel). Of the 12 samples screened here, we found sample C3 (Fig. 3A) displayed the most intense staining, samples C2, C4, C6, and C7 exhibited moderate expression (Fig. 2C and Fig. 3A) and low expression was visible in samples C9 and C11 (Fig. 3A and Fig. 3B). Furthermore, ORF1p positive tissues revealed that in sample C3 (high expression), ORF1p appeared predominantly in the nucleus (Fig. 3A).

Likewise, sample C2 and C4 (moderate expression) also showed nuclear staining with anti-ORF1p (Fig. 2C and Fig. 3). In contrast, in low expressing samples, our IHC indicates that ORF1p is mainly cytoplasmic. To complement our IHC analysis, we performed western blot analysis using total protein lysate from patient matched normal and OSCC tissue. Consistent with the IHC for sample 3, we detected a robust ORF1p band in cancer lysate (Fig. 3C, lane C3) while no band was observed in the lane loaded with lysate from normal tissue (Fig. 3C, lane N3). Protein lysate from MCF-7 cells served as a positive control (Fig. 3C, lane MCF-7). These data suggest that in our limited number of operated oral cancer samples ($n = 12$), around 60% showed L1 ORF1p expression.

Aberrant TP53 expression in oral carcinoma

Our knowledge of the mechanisms regulating L1 retrotransposition activity is incomplete. A recent study reported a positive correlation between TP53 mutation and L1 protein expression in several types of tumors [39,52]. Indeed *Rodic* et al. [39] demonstrated that the aberrant expression of TP53 is strongly associated with L1 expression in lung, ovarian, and pancreatic carcinoma. Analysis of TP53 expression in ORF1p positive oral carcinoma samples, we detected TP53 by IHC in three out of the four of the IHC positive samples (C3, C4, C6, and C11) but not an ORF1p negative sample (C10) (Fig. 4). Furthermore, no ORF1p or TP53 was detected in normal buccal mucosa tissue from patient number one (N1) (Fig. 4). These data suggest that L1 ORF1 protein expression in OSCC might have some link with aberrant TP53 expression.

Discussion

L1 protein expression is associated with hypomethylation of the L1 promoter in OSCC

A hallmark of cancer is an altered epigenetic landscape, which includes global hypomethylation and locus-specific hypermethylation [31,53–55]. Recent studies have reported that in many cancers, the L1 and Alu retrotransposons are heavily hypomethylated and thereby considered a surrogate marker for global DNA hypomethylation [21,22,35–37]. Our bisulfite analysis (Fig. 1) with OSCC samples indicates hypomethylation of the L1 5′-UTR, the region responsible for the L1 promoter activity. Notably, of the eight paired normal cancer samples assayed here, four displayed both significant hypomethylation throughout the CG-rich L1 promoter and upregulation of L1ORF1p. Previous work with oropharyngeal squamous cell carcinoma (OPSCC) samples highlights that the hypomethylation of the L1 promoter is extremely common and can be used as a marker to assess the risk of early post-treatment relapse [21]. The same study also showed the L1 promoter hypomethylation is increased in cases of HPV 16 negative samples. Perhaps, HPV16 infection contributes in some manner to L1 promoter methylation and L1 transcription [21]. The fifteen OSCC samples studied here were not checked for HPV infection; therefore, it is unknown if HPV infection has any correlation with L1-ORF1p expression. Further, studies will elucidate the impact of L1 hypomethylation on L1 protein expression and the number of new insertions events in cancers like OSCC.

ORF1p expression is common in OSCC

It has long been hypothesized that L1 retrotransposition activity may contribute to the onset and progression of cancer [53]. Only recently, with advances in DNA sequencing and effective reagents (e.g., antibodies reactive against L1 proteins), have significant developments been made in our understanding of L1 biology in cancer. Indeed, several recent studies have demonstrated that L1 retrotransposition is quite common in human cancers [39,49–51]. Likewise, it has been shown that about half of common cancers express human L1 ORF1p [36,39]. Here, we build on these studies by characterizing L1 ORF1p expression in OSCC samples. In addition to a dearth of data for L1 expression in OSCC, we chose to focus on this subtype of head and neck cancer because of its high prevalence, particularly among Indian patients [23–25]. Our analysis of 12 patient samples revealed that ~60% (7/12) are positive for L1 ORF1p expression. These data are in agreement with previous characterization of head and neck cancer samples, which identified ~61% positive for ORF1p [39].

Although localization studies have primarily observed ORF1p in the cytoplasm, a limited number of cells do occasionally display nuclear localization [47–51]. In the IHC analysis with cancer tissues, we observe ORF1p in both the nucleus and the cytoplasm. Specifically, we see strong staining of nuclear ORF1p in three (C2, C3 and C4) out of the 12 samples tested (Fig. 2C and Fig. 3A). Several recent studies have reported nuclear localization of L1ORF1p [57–60]. While ORF1p is absolutely required for retrotransposition *in cis* [7], it is unclear currently whether increased nuclear localization of ORF1p is associated with an increase in insertion frequency. Interestingly, studies of breast cancer using murine models and human samples have reported L1 proteins (ORF1p and ORF2p) in the nucleus in advanced stages of cancer [49–51]. Importantly, it has been reported that the samples associated with nuclear localization of L1 proteins showed very poor clinical outcome [49].

Tumor suppressor p53 protein might have some role in L1 activation in OSCC

Mutations in *p53* and its aberrant expression are common in almost every type of cancer [56,61]. Recent reports have shown that p53 can restrain retrotransposons and that this activity is evolutionarily conserved [52]. *Rodic* et al. [39] reported that upregulation of ORF1p in cancer tissues is correlated with highly expressed mutant p53. Consistently we observe elevated p53 expression in only ORF1p positive samples but not tissues where ORF1p was below the level of detection. Together, these data warrant further investigation into a potential role of mutant p53 might in L1 retrotransposition in OSCC. Although future studies, including L1 insertion analysis (e.g. L1-seq), will address whether L1 contributes to OSCC genome evolution, alternative functions for ORF1p, including its RNA-binding activity independent of retrotransposition in OSCC should not be dismissed.

Funding

This work was supported by a grant to PKM from the Department of Science and Technology (DST), India (grant no. EMR/2014/000167).

Author's contributions

SB conducted all the experiments and helped to write the manuscript. DS helped in Western, Immunohistochemistry and Immunocytochemistry techniques. SP helped in Sample collection and genomic DNA purification. KM reanalyzed the methylation data and helped to prepare the manuscript for resubmission. JN provided all the paired cancer- normal OSCC samples. SS helped in immunohistochemistry and analyzing data. MB and RP helped with resources and reviewing the data. PKM conceived of the study, supervised experiments, analyzed data and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We thank Dr. Biplab Bhattacharya and Dr. Jayita Bhattacharjee, Department of Earth Science IIT Roorkee, for helping with microscopy. We thank Dr. Sudha Bhattacharya (School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India) for helping with reagents and chemicals required in this study. We thank Chromgene Biotech Private Limited, Bengaluru, Karnataka, India, for helping with Sanger Sequencing. S.B is a recipient of a research fellowship from DBT, India. D.S and S.P and K.M are the recipients of the research fellowship from MHRD, India.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2020.01.050.

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