Nucleotide variants and protein expression of *TP53* in a Sri Lankan cohort of patients with head and neck cancer

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Abstract. Head and neck cancer (HNC) is the leading cancer in Sri Lankan males and second most common cancer among Sri Lankan females. This is the first study, to the best of our knowledge, that has focused on investigating the association between *TP53* somatic DNA variants, with p53 protein expression and risk factors in a cohort of Sri Lankan patients with HNC. A total of 44 patients with cancer and 20 healthy controls were studied. In total, 36 genomic DNA sequence variants were found, including several novel variants (two deletions in exons 4 and 6, two in the 3' untranslated region and several intronic variants). A total of 14 tumour samples carried pathogenic *TP53* mutations. A random selection of 24 samples was analysed immunohistochemically for p53 protein expression. All the samples with point missense variants were strongly immuno-positive, whereas, samples with nonsense and frameshift *TP53* variants were immuno-negative for p53 immunohistochemical staining. Although, the human papilloma virus is a known risk factor for HNC, results from the present study identified an absence or lower level of infection in the Sri Lankan cohort.

Introduction

Head and neck cancer (HNC) is a broad spectrum of disease that encompasses malignancies in the aero-digestive tract, including the oral cavity, pharynx, larynx, nasal cavity, paranasal sinuses, salivary glands, thyroid and parathyroid glands (1). HNC is the sixth most common cancer worldwide. Sri Lanka is considered as one of the high-risk countries, where HNCs are the leading cancer type in men accounting for 29% of all cancers (2-4).

Cigarette smoking and alcohol consumption, which are considered to be the most common risk factors for HNC, are generally lower in Sri Lankans compared to Western populations (2,5). However, tobacco chewing and consumption of areca nut are considered to be the most common causes of HNC in Sri Lankan males (3,5-7). Tobacco chewing results in exposure to 28 known carcinogens, including the non-volatile alkaloid-derived tobacco-specific N-nitrosamine and N-nitrosamino acids, which are different from the carcinogens involved in smoking cigarettes (3,8). The high risk associated with human papilloma virus (HPV) infection for specific types of HNCs is well established and studied in different geographical areas (9). However there is a scarcity of literature available on HPV associated HNC in the Sri Lankan population.

p53 acts as a 'guardian of the genome' to maintain the balance of cell death and proliferation by regulating the cell cycle, DNA repair, apoptosis, cellular metabolism and senescence (10). It is altered in ~50% of cancers overall and is more frequent in adult vs. childhood malignancies (11). The efficacy of many cancer therapeutic approaches is influenced by the functional status of the p53 tumour suppressor protein. Thus identification of *TP53* mutation status prior to administration of therapy can predict potential effectiveness of the treatment and influence treatment selection. Furthermore, the *TP53* mutation spectrum provides information on tumour origin, cause of mutation, aetiology, molecular pathogenesis, prediction of patient survival and chances of recurrence (12-15).

There were numerous studies on *TP53* variants in various cancers including head and neck cancer over the last few decades, particularly in Western populations. But there are only few studies done considering all subsets of HNC in Asia including India (16) and Japan (17) excluding Sri Lanka. Since the frequency of *TP53* mutations and the mutation spectra vary in different geographic areas, according to

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Abbreviations: DNE, dominant negative effect; HNC, head and neck cancer; HPV, human papilloma virus; IHC, immunohistochemistry; PCR, polymerase chain reaction; UTR, untranslated region

Key words: HNC, TP53, nucleotide sequencing, IHC, risk factors, Sri Lankan population

aetiological factors, life style, dietary pattern and culture, the present study has focused on establishing the *TP53* mutation spectrum in Sri Lankan HNC patients. Furthermore we used immunohistochemistry (IHC) to assess p53 protein expression and correlated immuno-expression of p53 with *TP53* gene mutational status. We also studied HPV infection in HNC and oesophageal cancer using p16 immuno-expression and HPV DNA detection, as the latter has reported to be associated with oral cancer in Sri Lankan patients (18).

Materials and methods

Patient recruitment and sample processing. Ethical approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka (EC/14/160). Patients with HNC (N=44) who had undergone surgical resection at the National Cancer Institute, Sri Lanka, were recruited for this study. Written informed consent from the study participants was obtained prior to recruitment. Socio-demographic and clinical data were obtained from study participants using questionnaires and by reviewing their medical reports. The majority of our patient population represents the Sinhalese ethnicity. Healthy controls (N=20; 10 males, 10 females) with no personal/family history of any cancer were recruited for this study.

Surgically excised tumour tissues were collected and the close adjacent region of the tissue section was placed in 10% formalin to prepare Formalin Fixed Paraffin Embedded tissue while the other section was immediately placed in Allprotect® Tissue Reagent (cat no. 76405; Qiagen, Hilden, Germany) and stored at -20˚C until processed. The hematoxylin and eosin stained slides of each tissue were reviewed by a pathologist to confirm the percentage of tumour region. Studied samples were with >50% area coverage of tumour in the study, except only two samples had <10% of tumour cells in the sections.

Genomic DNA was extracted from the excised tumour tissue of patients and from peripheral venous blood of healthy controls. Disruption of tissue specimens was done in liquid nitrogen using a motor and pestle followed by homogenization using QIAshredder (cat. no. 79654; Qiagen). Tissue DNA was extracted from homogenized sample using an All prep DNA/RNA/Protein mini kit (cat. no. 80004; Qiagen) following the manufacturer's protocol and stored at -20˚C until used. Genomic DNA was extracted from blood using the modified protocol described by Miller *et al* (19).

Seven sets of primers covering the entire exon 2-11 coding regions and adjacent flanking 5' and 3' intronic regions were designed using the online NCBI/Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=NM_001618.3). Polymerase Chain Reaction (PCR) amplification was performed using each primer set in a final volume of 25 μl containing 100 ng genomic DNA, 3.5 mM $MgCl_2$, 1X Green GoTaq® reaction buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], 2.5 mM dNTPs (Promega Corporation, Madison, WI, USA), 5 pmols of each primer (IDT Integrated DNA Technologies, Coralville, IA, USA) and 1 unit of GoTaq® Flexi DNA polymerase (Promega Corporation). PCR conditions: 94˚C for 7 min, followed by 33 cycles of 94˚C for 1 min, at the optimized annealing temperature for 1 min and 72˚C for 1 min and a final extension step of 72˚C for 10 min was performed in a thermocycler (Veriti Thermal Cycler; Thermo Fisher Scientific, Waltham, MA USA). The annealing temperature and $MgCl_2$ concentration were optimized for each primer set. The primer nucleotide sequences, amplicon sizes and annealing temperatures are shown in Table I.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega Corporation) and purified products were directly sequenced using the BigDye® Terminator v3.1 kit (Thermo Fisher Scientific) and an Applied Biosystems™ 3500Dx Genetic Analyzer (Thermo Fisher Scientific). Sequence variants detected were reconfirmed by performing a second PCR and direct sequencing.

Sequencing results were analysed to identify variants by alignment with a human *TP53* NCBI reference sequence (GenBank accession number-NC_000017), via Bio Edit® software and further confirmed by Mutation Surveyor® v4.0.9 and Alamut® Visual 2.7.2 Documentation. Identified sequence variants were named according to the Human Genome Variation Society/HGVS nomenclature guidelines (http://www.hgvs.org/mutnomen/).

Variant analysis. Identified sequence variants were checked for previous reports in the following databases: Catalogue Of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic); NCBI (https://www.ncbi.nlm.nih.gov/); IARC TP53 (http://p53.iarc.fr/); Ensembl (https://asia.ensembl.org/index.html); the p53 website (https://p53.fr/tp53-database).

Pathogenicity of the identified exonic variants was analysed using five comparative missense prediction programs: Align GVGD (http://agvgd.hci.utah.edu/agvgd_input.php); SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html); MutationTaster (http://www.mutationtaster.org/); PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/); Provean (http://provean.jcvi.org/seq_submit.php). p53 specific structural and functional activity (transcriptional activity, dominant negative effect) data available on IARC TP53 database was also considered while determining the pathogenicity of the identified variants (18,19). Human Splicing Finder V3.0 (http://www.umd.be/HSF3/) and splicing window of Alamut® Visual software, integrating a number of prediction methods (for splice signal detection: MaxEntScan, GeneSplicer and ESE/exonic splicing enhancer binding site detection: ESEFinder, RESCUE-ESE) were used to assess the impact on gene splicing of identified intronic variants. All variants were classified according to American College of Medical Genetics standards and guidelines (20).

Immunohistochemistry for p53. IHC characterization of p53 expression was performed on twenty four randomly selected representative formalin fixed paraffin-embedded tumour tissue sections. The primary antibody used was mouse monoclonal Anti-Human p53 clone DO-7 (Agilent DaKo, Santa Clara, USA) at 1:100 dilution. Tissue sections of 4 micron thickness were mounted on microscopic slides and dried at 60˚C for 2 h. After dewaxing in xylene, sections were rehydrated in graded (100, 95, 70 and 60%) alcohol. Microwave heating in citrate (pH 6) buffer was used for antigen retrieval and endogenous peroxidase was inhibited by incubating tissue sections in freshly prepared 3% hydrogen peroxide. Tissue sections were

Table I. Nucleotide sequence of primers used for amplification of TP53, amplicon size and annealing temperature.

Exon	Primer sequence, 5'-3'	Amplicon size (bp)	Annealing temperature (˚C)
2 and 3	CAGCCATTCTTTTCCTGCTC GGGGACTGTAGATGGGTGAA	497	62
4	CCTGGTCCTCTGACTGCTCT GCCAGGCATTGAAGTCTCAT	361	64
5 and 6	GTTTCTTTGCTGCCGTCTTC CTTAACCCCTCCTCCCAGAG	500	64
7	GAGCTTGCAGTGAGCTGAGA TCCCAAAGCCAGAGAAAAGA	444	63
8 and 9	CAAGGGTGGTTGGGAGTAGA TGTCTTTGAGGCATCACTGC	532	65
10	TGCATGTTGCTTTTGTACCG GAAGGCAGGATGAGAATGGA	299	56
11	TGTCATCTCTCCTCCCTGCT AAGTGGGCCCCTACCTAGAA	438	61

incubated with primary antibody at room temperature for an hour and then exposed to horseradish peroxidase (A. Menarini Diagnostics Ltd., Winnersh, UK) conjugated anti-mouse IgG secondary antibody for 30 min. Universal probe (A. Menarini Diagnostics Ltd.) was applied for 30 min before the addition of primary antibody, in order to increase the staining sensitivity 10 to 40 times for mouse monoclonal antibodies. Then, the slides were incubated with 3,3'-diaminobenzidine (A. Menarini Diagnostics Ltd.). Following the incubation, the sections were washed and counter stained with haematoxylin. Slides that were not incubated in primary antibody were used as antibody negative controls to check for specificity of staining.

Images of the stained slides were visualized using the AperioScanScope® CS System, an automated digital scanner (Aperio Technologies, Bristol, UK) technology and Spectrum™ image management software. The slides were analysed by both automated and manual methods, blinded from the clinical data.

Human papilloma virus DNA screening. PCR with $GP5^+/GP6^+$ HPV specific primers was performed for all HNC samples in a final volume of 25 μl containing 100-150 ng genomic DNA, 3.5 mM $MgCl_2$, 1X Green GoTaq® reaction buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], 2.5 mM dNTPs (Promega Corporation), 50 pmols of each primer (IDT Integrated DNA Technologies) and 1 unit of GoTaq® Flexi DNA polymerase (Promega Corporation). PCR conditions: 94˚C for 4 min, followed by 40 cycles of 94˚C for 1 min, 40˚C for 1 min and 72˚C for 1 min and a final extension step at 72˚C for 10 min (21). The negative control included all reagents except for DNA. A p1203 PML2d HPV-16 plasmid (gift from Peter Howley: Addgene plasmid #10869) was used as the positive control. Samples positive for HPV generate a 140-bp-long fragment from the HPV L1 structural gene.

p16 immunohistochemistry. p16 cyclin-dependent kinase inhibitor protein expression was detected using IHC at the pathology laboratory of the Royal Victoria Infirmary, Newcastle upon Tyne using a Ventana Benchmark XT Automated IHC/*In situ* hybridization slide staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA). The CINtec® p16 Histology (Hoffmann-La Roche AG, Basel, Switzerland) and UltraView DAB detection kit (Ventana Medical Systems Inc.) were used for the detection of p16. Images of the stained slides were visualized and analysed using the same process as for p53 detection.

Patient follow-up. Data on response to treatment, survival, recurrence and current status during the follow-up period were collected by reviewing patient medical records and collecting data directly from patients/guardians.

Statistical analysis. All categorical data were analysed using Fisher's exact test to assess the significance of the associations. A P-value <0.05 was considered as statistically significant.

Results

Baseline characteristics of participants. Out of the 44 patients, 75% (N=33) were males and 25% (N=11) were females. Mean ± SD age was 59.03±11.68 years for males and 53.27±19.04 years for females. Healthy controls were younger (males 33.2±4.92; females 33.1±5.84 years). Table II summarizes the characteristics of the patients, tumour type and possible risk factors the patients were exposed to.

Analysis of TP53 sequence variants and epidemiological/clinical correlations. A total of 36 sequence variants (18 translated exonic variants, 12 intronic and 2 non-translated exonic variants) were found in 44 patients and 4 additional sequence variants were found only in healthy controls (two exonic silent and two novel intronic variants). Table III illustrates the characteristics, *In silico* and functional analysis of each variant. All the exonic variants were in the DNA binding domain of the p53 protein (codons 94-292).

c.298delC/p.Gln100Argfs*23, a novel heterozygous frameshift variant in exon 4 that created a stop codon at position 123, was found in a male patient with upper oesophageal cancer (Fig. 1) diagnosed at 51 years of age. A c.383delC/p.Pro128Leufs*42 frameshift variant in exon 5 that created a stop codon at position 170 was also found in a male patient with cancer recurrence in the vocal cord.

A novel 12 base pair in-frame deletion in exon 6 (c.626_637delGAAACACTTTTC/p.Asn210_Arg213del), that would results in the loss of 4 amino acid residues from 210 to 213, producing a 389 amino acid protein (Fig. 2) was detected in the tumour DNA of a 61-year-old male patient with oesophageal cancer.

Another male patient with recurrent cheek cancer had one nonsense pathogenic variant, c.493C>T (p.Gln165*) in exon 5, creating a stop codon at position 165, together with a pathogenic missense variant (c.578A>G/p.His193Arg) in exon 6 and survived only two years and eight months following recurrence. A c.637C>T (p.Arg213*) a nonsense pathogenic variant in exon 6, creating a stop codon at 213 position was

Table II. Baseline characteristics of the patients, tumour type and possible risk factors the patients were exposed to.

Characteristics	Total number of patients	Patients with wild-type *TP53, no.*	Patients with mutated *TP53, no.*	Total number of healthy controls	Two-tailed P-value between patient and healthy controls[a]
Sex					
Male	33	23	10	10	0.1176
Female	11	7	4	10	
Ethnicity					
Sinhalese	35	23	12	13	0.0888
Sri Lankan Tamil	3	3	0	5	
Indian Tamil	3	1	2	1	
Muslims	3	3	0	0	
Burghers	0	0	0	1	
Age at study entry					
<30 years	2	2	0	2	0.0007
30-60 years	24	14	10	18	
>61 years	18	14	4	0	
Tumour type					
Squamous cell carcinoma	25	13	12	N/A	N/A
Adenocarcinoma	4	3	1	N/A	
Papillary carcinoma	4	4	0	N/A	
Others	11	10	1	N/A	
Smoking history					
Yes	17	11	6	0	0.0005
No	25	17	8	20	
Unknown	2	2	0	0	
Alcohol consumption					
Yes	13	8	5	1	0.0251
No	29	20	9	19	
Unknown	2	2	0	0	
Betel-quid chewing					
Yes with tobacco	21	12	9	0	<0.0001
Yes without tobacco	2	2	0	0	
No	19	14	5	20	
Unknown	2	2	0	0	
Roofing					
Asbestos	21	14	7	1	<0.0001
Tile	15	11	4	5	
Concrete	1	1	0	12	
Metal	3	2	1	0	
Unknown	4	2	2	2	
Codon 72 polymorphism					0.6225
Arginine	10	6	4	7	
Proline	19	12	7	7	
Arginine/proline	15	12	3	6	

[a]Fisher's exact test was done to examine the significance of the associations between patients and healthy controls. A P-value <0.05 was considered as statistically significant.

observed in a 72-year-old male patient with tumour of the mandible.

A 59-year-old female patient with a malignancy in the cheek carried two variants in exon 5; a pathogenic missense variant (c.422G>T/p.Cys141Phe) and a likely-pathogenic missense variant (c.467G>A/p.Arg156His). She survived for one year and eight months following diagnosis.

Another 59-year-old male patient had a cancer diagnosed in 2010 in the retro molar region which recurred in the mandible after 5 years. He had a single pathogenic missense variant, c.455C>T/p.Pro152Leu in exon 5. Another pathogenic variant, c.524G>A/p.Arg175His in exon 5 was observed in a male patient with left antral malignancy and in a female patient with oesophageal cancer.

Table III. *In silico* and functional analysis of identified variants.

No.	HGVS Nomenclature		Location	Mutation type	No. of carriers in the study cohort		Code reported in databases	*In silico* prediction								Prediction of functional activity			Conclusion
	cDNA	Protein			Patients (n=44)	Controls (n=20)		Splice site	CpG site	Align GVGD	Mutation taster	Provean	SIFT	Poly Phen 2	NCBI	Transcriptional activity [21]	Functional activity prediction [22]	Dominant negative effect	
1	c.298delC	p.Gln100Argfs*23	E4	FS	1	0	Novel	-	-	-	DC	-	-	-	-	-	-	-	Path
2	c.383delC	p.Pro128Leufs*42	E5	FS	1	0	COSM5198771	-	-	-	DC	-	-	-	-	-	-	-	Path
3	c.626_637del GAAACAC TTTTC	p.Asn210_Arg213 del	E6	IF	1	0	Novel	-	-	-	DC	-	-	-	-	-	-	-	Path
4	c.493C>T	p.Gln165*	E 5	NS	1	0	rs730882001, COSM43632	-	-	-	DC	D	-	-	Path	-	-	-	Path
5	c.637C>T	p.Arg213*	E 6	NS	1	0	rs397516436	-	Yes	-	-	-	-	-	Path	-	-	-	Path
6	c.422G>T	p.Cys141Phe	E 5	M	1	0	COSM44911	-	-	C65	DC	D	D	PRB	-	PF	F	-	Path
7	c.455C>T	p.Pro152Leu	E5	M	1	0	rs587782705	-	Yes	C65	DC	D	D	POB	Path	NF	NF	ND	Path
8	c.524G>A	p.Arg175His	E 5	M	2	0	rs28934578, COSM10648	-	Yes	C25	DC	D	D	POB	Path	NF	NF	DN	Path
9	c.578A>G	p.His193Arg	E 6	M	1	0	rs786201838, COSM10742	-	-	C25	DC	D	D	PRB	LP	NF	NF	MD	Path
10	c.583A>T	p.Ile195Phe	E6	M	1	0	COSM44633	-	-	C0	DC	D	D	PRB	-	NF	NF	-	Path
11	c.646G>C	p.Val216Leu	E 6	M	1	0	rs730882025	-	-	C25	DC	-	D	-	LP	NF	NF	-	Path
12	c.659A>G	p.Tyr220Cys	E6	M	1	0	rs121912666, COSM10758	-	-	C65	DC	D	D	PRB	Path	NF	NF	MD	Path
13	c.747G>T	p.Arg249Ser	E7	M	1	0	rs28934571, COSM10817	-	-	C65	DC	D	D	PRB	Path	NF	NF	DN	Path
14	c.818G>A	p.Arg273His	E 8	M	1	0	rs28934576, COSM10660	-	Yes	C25	DC	-	D	-	Path	NF	NF	DN	Path
15	c.844C>T	p.Arg282Trp	E 8	M	1	0	rs28934574, COSM10704	-	Yes	C65	DC	D	D	PRB	Path	NF	NF	MD	Path
16	c.467G>A	p.Arg156His	E 5	M	1	0	rs371524413, COSM43739	-	Yes	C0	P	N	T	POB	LP	PF	NF	ND	LP
17	c.576G>C	p.Gln192His	E 6	M	2	0	COSM44554	-	-	C0	DC	N	T	B	-	F	F	-	LB
18	c.648G>C	p.Val216Val	E 6	S	1	0	rs199693249	-	-	-	-	-	-	-	-	-	-	-	LB
19	c.63C>T	p.Asp21Asp	E 2	S	0	1	rs1800369	-	-	-	-	-	-	-	-	-	-	-	LB
20	c.459C>T	p.Pro153Pro	E 5	S	0	1	rs72661116	-	-	-	-	-	-	-	LB	-	-	-	LB
21	c.-140G>A	-	E 1	3'UTR	4	0	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
22	c.-159delT	-	E 2	3'UTR	1	0	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
23	c.97-29C>A	-	I 3	I	4	2	rs17883323	Al	-	-	-	-	-	-	-	-	-	-	US
24	c.994-95 delT	-	I 9	I	4	0	Novel	Al	-	-	-	-	-	-	-	-	-	-	US
25	c.74+16G>C	-	I 2	I	2	0	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
26	c.74+38C>G	-	I 2	I	25	12	rs1642785	-	-	-	-	-	-	-	-	-	-	-	LB
27	c.96+41_96+56 delACCTGG AGGGCTG GGG	-	I 3	I	39	19	rs59758982	-	-	-	-	-	-	-	-	-	-	-	LB
28	c.97-52G>A	-	I 3	I	1	0	rs540683791	-	-	-	-	-	-	-	-	-	-	-	LB
29	c.375+37C>A	-	I 4	I	1	0	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
30	c.782+72C>T	-	I 7	I	22	9	rs12947788	-	-	-	-	-	-	-	-	-	-	-	LB
31	c.782+92T>G	-	I 7	I	22	9	rs12951053	-	-	-	-	-	-	-	-	-	-	-	LB
32	c.993+12T>C	-	I 9	I	1	0	rs1800899	-	-	-	-	-	-	-	-	-	-	-	LB
33	c.1100+76T>A	-	I 10	I	2	0	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
34	c.75-42G>A	-	I 2	I	0	1	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
35	c.782+79C>T	-	I 7	I	0	1	Novel	-	-	-	-	-	-	-	-	-	-	-	LB
36	c.673-36G>C	-	I 6	I	2	0	rs17880604	-	-	-	-	-	-	-	B	-	-	-	B

Variants were classified as 'non-functional' if the median is ≤20, 'Partially functional' if the median is >20 and ≤75, 'functional' if the median is >75 and ≤140, and 'supertrans' if the median is >140 based on the median of 8 promoter-specific activities, expressed as percent of wild-type protein and were categorized as 'DNE' if they were Dominant Negative on both $p21^{WAF1}$ and RGC promoters or on all other promoters, 'moderate-DNE' if they were Dominant Negative on some promoters and not on others, and 'non-DNE' if they were not Dominant Negative on both WAF1 and RGC promoters, or none of the promoters [23, 24]. DC, disease causing; P, polymorphism; N, neutral; t, tolerated; PRB, probably damaging; POB, possibly damaging; path, pathogenic; LP, likely pathogenic; US, variant with uncertain significance; LB, likely benign; B, benign; FS, frameshift; IF, in-frame; M, missense; NS, nonsense; S, silent; I, intron; E, exon; 3'UTR, 3' untranslated region; F, functional; PF, partially functional; D, deleterious; NF, non-functional; Al, alter the splice site; DN, dominant negative effect; MD, moderate dominant negative effect; ND, non dominant negative effect.

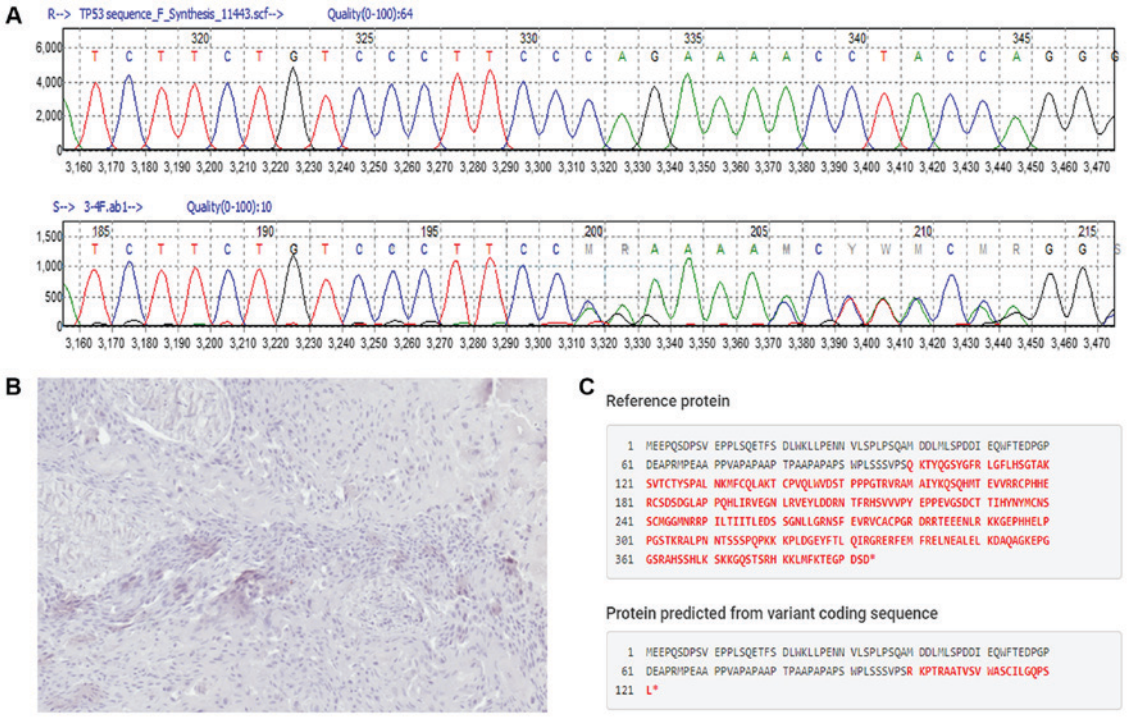

Figure 1. Novel frameshift variant c.298delC/p.Gln100Argfs*23 detected in Exon 4. (A) Mutation Surveyor®V4.0.9 image indicating the one base pair heterozygous deletion point; R indicates the reference *TP53* sequence and S indicates the study sample *TP53* sequence. (B) p53 immunohistochemical staining of the tumour sample with the above mutation. Magnification, x20. (C) Protein prediction using Mutalyzer 2.0.26.

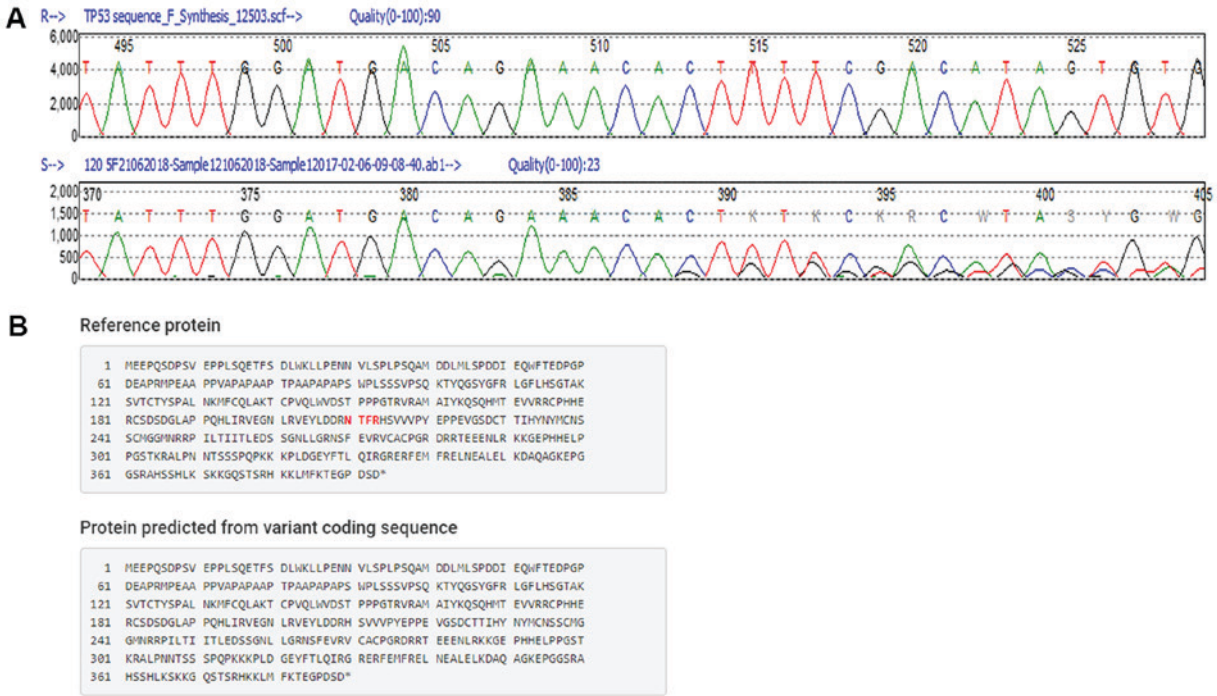

Figure 2. Novel in-frame deletion c.626_637delGAAACACTTTTC/p.Asn210_Arg213del detected in exon 6. (A) Mutation Surveyor®V4.0.9 image indicating the 12-base pair heterozygous deletion; R indicates the reference *TP53* sequence and S indicates the study sample *TP53* sequence. (B) Protein prediction using Mutalyzer 2.0.26.

Four pathogenic missense variants were detected in exon 6 (c.583A>T, c.659A>G, c.646G>C, c.578A>G). One male patient aged 59 diagnosed with upper oesophageal cancer had a c.583A>T/p.Ile195Phe variant. A c.659A>G/p.Tyr220Cys variant was found in a 55-year-old male patient with malignancy in the retro molar region. This variant co-existed with another pathogenic variant, c.818G>A/p.Arg273His, in exon 8. Co-existence of c.646G>C and c.648G>C (p.Val216Leu) variants were identified in the malignant cheek tumour of a 60-year-old female patient.

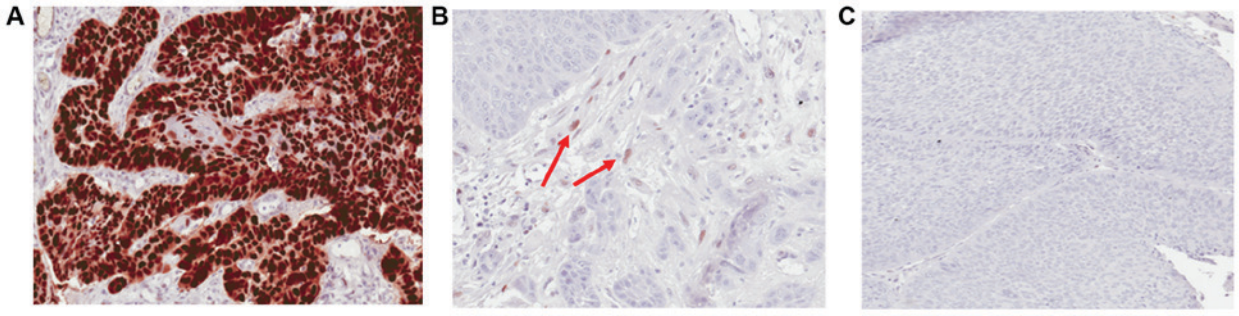

Figure 3. IHC characterization of p53 detected by DO7 antibody. (A) Pattern A with wide spread IHC positive nuclei-IHC score >20. (B) Pattern B with rare positive single tumour nuclei-IHC score from ≥1 to ≤20; arrows indicate the rare positive cells. (C) Pattern C with no IHC positive nuclei-IHC score from 0 to <1. Magnification, x20. IHC, immunohistochemistry.

c.576G>C/p.Gln192His, a likely-benign variant was identified in exon 6 in a 68-year-old female patient with oesophageal cancer and in a male patient with arytenoid cartilage cancer. The only pathogenic missense variant c.747G>T/p.Arg249Ser in exon 7 was observed in a male patient aged 68 years with cancer in the cheek.

A c.844C>T/p.Arg282Trp variant in exon 8 was found in a 60-year-old female patient with a mandible cancer who survived only 1 year and 5 months following diagnosis.

Among the 14 patients who had pathogenic variants, eight had a history of betel quid-tobacco chewing, five in the absence of smoking or alcoholism and three in the presence of smoking and alcoholism. Six of the patients with pathogenic variants did not have a history of betel quid-tobacco chewing, but three of them were smokers and had a history of alcoholism.

Among the intronic variants, c.994-95delT, a novel deletion in intron 9 was identified in 4 patients but not in controls and c.97-29C>A, a reported variant substitution in intron 3, which was identified in four patients and in two controls were categorized as variants with uncertain significance as both alter the WT branch point and potentially alter the splicing according to Human Splicing finder.

Two novel variants were found in the 5'UTR of the gene; c.-140G>A (N=4) and c.-159delT (N=1) in patients but not in healthy controls. Three more novel intronic variants were found in patients [c.74+16G>C (N=2), c.375+37C>A (N=1), c.1100+76T>A (N=2)] in introns 2, 4 and 10 respectively but not in any healthy controls. In contrast, two other novel variants [c.75-42G>A (intron 2) and c.782+79C>T (intron 7)] were observed in healthy controls but not in patients.

TP53 gene status and expression of p53 protein. Twenty four samples were randomly chosen for IHC analysis. The H-Score for each slide was calculated by multiplying the intensity of staining (no staining, 0; weak, 1; intermediate, 2; and strong, 3) by the percentage of cells at each staining intensity level. Scores ranged from 0 to 300. Scores were grouped into three categories; from 0 to <1 was considered negative (Pattern-C), score ranging from 1 to 20 (Pattern-B) and all the scores >20 (Pattern-A) (Fig. 3).

IHC staining for p53 showed positive immuno-reactivity in 13/24 (54.17%) cases. Nine of these tumours showed widespread IHC positive tumour nuclear staining involving either the entire tissue section or a segment of it (Pattern-A) while 4 showed rare/scattered p53 positive single tumour nuclei (Pattern-B). All cases with a missense variant in *TP53* gene showed Pattern-A IHC staining. However, vice versa is not true because 2 cases showed IHC positivity with Pattern-A in the absence of detectable pathogenic *TP53* gene variants. These had only ~10% of positively stained tumour cells in the respective tissue sections. One case with both missense and nonsense variants showed Pattern-A IHC staining. All 4 cases with Pattern-B IHC staining had no detectable pathogenic *TP53* variants. Among the 11 cases with immuno-negativity (Pattern-C), two had frameshift variants and one had a nonsense variant. The remaining eight patients who showed immuno-negativity had wild-type *TP53*.

Prevalence of HPV. Sensitivity of the $GP5^+/GP6^+$ primer pair was at the level of femtograms for HPV genotypes which match strongly with the primers and at the level of picograms for HPV genotypes having four or more mismatches to the primers (21). Absence of positive results in our study cohort indicated either absence of HPV DNA or the presence of HPV DNA at a concentration below the level of sensitivity.

The IHC staining for p16 is defined as positive if there is strong and diffuse nuclear and cytoplasmic staining pattern in >70% of the tumour specimen (Fig. 4). Regardless of cancer type, *TP53* mutation status and p53 protein expression, all HNC samples in the Sri Lankan study cohort were negative for p16 staining, despite clear positive staining with the control sample.

Discussion

This is a preliminary study on Sri Lankan HNC patients, focused on the mutation spectrum of the *TP53* gene, expression of p53 protein and relationship to possible risk factors. In the present study, we also included upper oesophageal cancer, as HNCs are often associated with oesophageal cancer and both tumour types share common risk factors (22).

Identification of hotspot regions of *TP53* mutations in the Sri Lankan context is useful to prioritize screening of such regions rather screening the entire gene, prior to treatment. In the current study, 70% (11 out of 15) of pathogenic mutations reported were detected in the exon 5 and 6 which is covered by a single set of primer. Therefore it is worth to understand the hotspot region in *TP53* for initiation of screening of *TP53* in a resource limited setting such as Sri Lanka. Hotspot

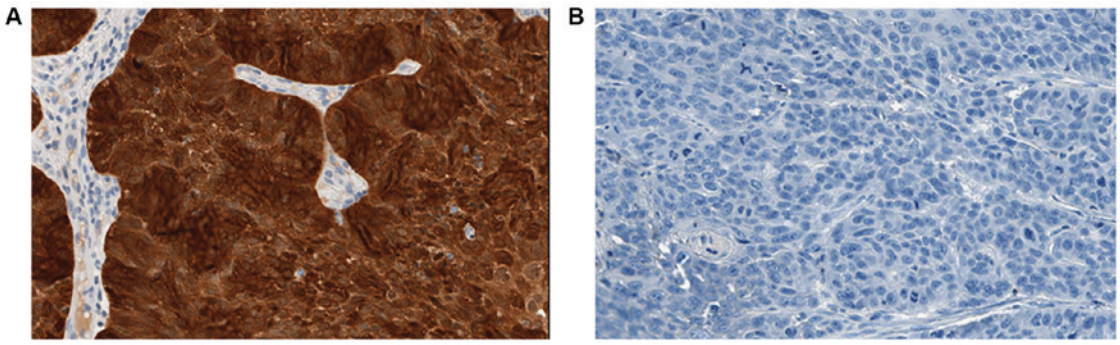

Figure 4. Detection of p16 expression using Ventana Benchmark XT Automated immunohistochemistry/*in situ* hybridization slide staining system. (A) Positive control showing positive staining of nuclei and cytoplasm. (B) A study sample with negative staining. Magnification, x20.

regions of *TP53* identified in the present study are similar to those reported for other ethnic groups. This is also supported by the data in the 'IARC TP53 database' (http://p53.iarc.fr/TP53SomaticMutations.aspx) and 'cBioPortal for cancer genomics' (http://www.cbioportal.org/).

Among the pathogenic variants found, the frameshift variant c.298delC and in-frame variant c.626_637delGAAACACTTTTC have not been previously reported. The c.646G>C somatic missense variant is reported only in the IARC TP53 database, but the details are not provided. c.383delC and c.576G>C are novel variants in HNCs, as they have been reported only in the COSMIC database for breast and stomach tumours respectively. Although c.422G>T, c.455C>T, c.467G>A, c.747G>T and c.818G>A have been commonly reported for many types of cancer, these variants have not been reported in HNC or oesophageal cancer.

Some mutations of *TP53* such as frame shift deletions or insertions and nonsense or splice site mutations are recessive and require loss of the remaining normal allele for cells to lose p53 tumour suppressor function. However, some point missense mutant forms can have a dominant-negative effect (DNE) because they are no longer recognised by their negative regulator MDM2 and accumulate in the cell to a much greater level than the normal wild-type *TP53* allele. These so-called 'dominant-negative' forms of mutant p53 nevertheless have lost their normal transcriptional activity. Loss of function of each identified variant in the current study was assessed based on the reported loss of transcriptional activity data available in the IARC and other databases. Overall transcriptional activity of 2314 distinct mutant proteins has been experimentally measured on a panel of p53-response elements of promoters of downstream transcriptional target genes such as *CDKN1A* (p21), *MDM2* and *BAX* (23-25). In addition sequence variants have also been evaluated using a computational geometry approach called Delaunay Tessellations to predict the functional impact (26). DNE of sequence variants has been assessed based on studies carried out on promoters of $p21^{WAF}$, Ribosomal Gene Clusters, etc. (24). Out of ten pathogenic missense variants reported in our study cohort, nine (c.455C>T, c.524G>A, c.578A>G, c.583A>T, c.659A>G, c.646G>C, c.747G>T, c.818G>A and c.844C>T) are predicted to involve complete loss of transcriptional function based on databases compiled from both yeast assay and computational method.

Codon 72 variant (p.Arg72Pro) in exon 4 is a well-known polymorphism present in the normal human population. The implication of this polymorphism in cancer risk and prognosis is controversial, with some earlier studies reporting that p53 protein containing the codon 72 Arginine form has a greater apoptotic potential (27-31) while others have failed to replicate these findings (32,33). In the present study neither the p.Arg72Pro alleles was significantly associated with cancer.

One previous study of *TP53* carried out in 1998 on 23 oral squamous cell carcinoma from Sri Lankan patients has been published. This reported nine sequence variants: Missense variant in codons 135, 164, 176, 245, 248; deletions in codons 130, 144-148, 172-187 and one insertion in codon 250 (34). None of these variants were found in the current study, which included 19 patients with oral squamous cell carcinoma. However studies done in all subset of HNC in India and Japan showed 21 and 11.6% of pathogenic *TP53* variant and 69 and 13.7% of HPV positivity respectively (16,17).

Generally, for head and neck cancers without HPV infection has a higher prevalence of *TP53* mutation. However, the lower percentage of *TP53* mutations was observed in the current study is due to the inclusion of thyroid cancer in the studied cohort as it is considered under head and neck cancer in Sri Lankan context. In the current study, out of 44 patients, 9 patients were with thyroid carcinoma and none of them carried any pathogenic *TP53* variants, which may be the possible reason for a decreased percentage of *TP53* mutation prevalence (35,36).

TP53 mutations can be classified as disruptive and non-disruptive based on the location and type of mutations. Disruptive mutations are missense mutations located in the DNA-binding domain, or stop codons in any region which create truncated proteins. Non-disruptive mutations are missense mutations located outside the DNA binding domain. Disruptive mutations are closely associated with poor prognosis of head and neck cancer (37) and all the reported pathogenic somatic *TP53* mutations in the current study are associated with poor prognosis.

However, we also analysed the pathogenic missense mutations with a computational approach called evolutionary action or EAp53, which has been validated both *in vivo* and *in vitro*, for *TP53* mutation classification (http://mammoth.bcm.tmc.edu/EAp53/). This approach assigns an evolutionary action score and classifies each p53 missense mutation as either high- or low-risk. According to Neskey *et al* (38), patients with high risk TP53 mutations show poor survival with high invasive and aggressive tumour behaviour. According to the EAp53 analysis c.422G>T, c.455C>T, c.524G>A, c.578A>G, c.747G>T were

categorized as high risk mutations and c.583A>T, c.646G>C, c.659A>G, c.818G>A, c.844C>T as low risk mutations. In addition all truncating mutations are also considered to have poor prognosis.

Generally wild-type p53 protein has very short half-life in the cells under normal conditions due to constant degradation by the MDM2-mediated negative feedback loop (39). In the case of mutant p53 protein, MDM2 protein cannot be induced, thus MDM2-mediated negative feedback is absent leading to accumulation of mutant p53 (40). It is also reported that a contribution to the stabilization of mutant p53 is due to impaired ubiquitination (41). All the missense variations identified in the current study showed the accumulation of p53 protein with the Pattern-A strong immunostaining. However the converse was not true, as there were two samples with Pattern-A IHC which showed no detectable variations in sequence. But these two samples have only <10% of tumour cells in the sections used for immunostaining which would have been below the sensitivity of detection by sanger sequencing.

All the truncated proteins that resulted either from frame-shift or nonsense variations showed immuno-negativity. The antibody used to detect the expression of p53 protein is DO-7 which binds to the p53 protein between amino acid 1-45 (42). Even though all four truncated protein are longer than 123 amino acids, they remained undetectable. This may be due to nonsense-mediated decay of mRNA with premature stop codon may have resulted in less stable truncated proteins.

All the samples (N=4) with Pattern-B IHC were *TP53* wild-type and, there were 57% (8/14) of the wild-type samples that showed no positive immuno-reactivity. Thus IHC analysis cannot be used as a standalone method to detect the alteration in p53 as it cannot differentiate between truncated p53 protein and wild-type p53 protein as both show an immuno-negative IHC pattern.

Cigarette smoking and alcoholism are well known triggers of HNCs. The awareness of these risk factors is higher among Sri Lankans. But despite a reduction in cigarette smoking, the burden of HNC has increased constantly over the years, which prompted us to search for other risk factors.

Betel-quid chewing with tobacco is a common habit among Sri Lankans especially those who are engaged in hard physical work for long hour. Furthermore, there is poor awareness of the harmfulness of this habit. 47.7% of the patients (N=21) studied were addicted to betel-quid chewing with tobacco and out of them, nine have *TP53* sequence variants conferring an altered protein structure.

HPVs are non-enveloped DNA viruses, containing a double stranded circular DNA which consists of an upstream regulatory region (URR), an early region (E) encoding viral regulatory proteins and a late region (L) encoding viral capsid proteins (43). HPV is an identified cause of HNCs and it is reported that HPV-positive HNCs have a better prognosis (44-46). The high-risk HPV proteins E6, E7 target tumour suppressor proteins p53 and RB respectively. Disruption of the function of p53 and pRB lead to uncontrolled cell division (47). Here we studied the expression of p16 protein which is used as a surrogate marker to identify functionally active HPV infection as it is reciprocally overexpressed due to functional inactivation of pRB by E7 protein (48,49). Several previous studies in other countries reported p16 overexpression in HNC. The highest (38%) and lowest (7.8%) level of p16 expression are reported in the USA, while intermediate levels are reported from India (20%) and Kenya (14.6%) (50-53). All samples in our study cohort were negative for p16 staining which may suggest the absence of functionally active HPV infection (54).

In a previous study from Sri Lanka 37.2% of tumour samples from oral cancer patients were reported to be infected when analysed using HPV DNA typing (18). However in the current study, none of the patients were positive on DNA typing which also indicates the absence or presence of very low concentration of HPV DNA in the tumour samples.

It has also been reported that p53 protein with the Arginine72 polymorphism is efficiently degraded by the E6 protein of HPV 16, thus increasing susceptibility to cancer (55). Nevertheless, there are studies that show no correlation between p.Arg72Pro and HPV related cervical cancers (56,57). Our cohort of HNC patients also showed no correlation between p.Arg72Pro and HPV infection.

Exposure to asbestos due to living in houses with asbestos roofing and exposure to rubber by occupations may also have contributed to mutagenesis of *TP53* and carcinogenesis in some patients in the present cohort.

In conclusion, we examined all exons and splicing sites of the *TP53* gene in HNC and oesophageal cancers from a cohort of Sri Lankan patients and found a high occurrence of gene alterations including several novel variants. All p53 protein altering variants found were positioned between exons 4-8. Only the point missense variants were associated with strong immuno-positivity, thus limiting the use of IHC in detecting mutation status of p53. Our cohort of patients did not appear to carry significant levels of HPV infection.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VM performed the experiments, contributed to the analysis and interpretation of the data, and drafted the manuscript. EHK, KHT and SDS conceived and designed the study, were involved in the molecular genetic studies, data analysis and interpretation, and the revision of the manuscript. KDS and PA provided clinical expertise, recruited the study participants, and supervised the clinical data and histopathological characterization. JL designed the immunohistochemical and HPV studies, and contributed to the analysis of the sequencing data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka (EC/14/160). Written informed consent form at the time of enrolment was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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