New insights from Whole Genome Sequencing: BCLAF1 deletion as a structural variant that predisposes cells towards cellular transformation

LAMECH M. MWAPAGHA[1,2], VIMBAISHE CHIBANGA[1,3], HENDRINA SHIPANGA[1] and M. IQBAL PARKER[1]

[1]Department of Integrative Biomedical Sciences, Division of Medical Biochemistry and Structural Biology, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, Western Cape 7925, South Africa

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Abstract. Cancer arises from a multi-step cellular transformation process where some mutations may be inherited, while others are acquired during the process of malignant transformation. Aberrations in the BCL2 associated transcription factor 1 (*BCLAF1*) gene have previously been identified in patients with cancer and the aim of the present study was to identify structural variants (SVs) and the effects of *BCLAF1* gene silencing on cell transformation. Whole-genome sequencing was performed on DNA isolated from tumour biopsies with a histologically confirmed diagnosis of oesophageal squamous cell carcinoma (OSCC). Paired-end sequencing was performed on the Illumina HiSeq2000, with 300 bp reads. Reads were aligned to the Homo sapiens reference genome (NCBI37) using ELAND and CASAVA software. SVs reported from the alignment were collated with gene loci, using the variant effect predictor of Ensembl. The affected genes were subsequently cross-checked against the Genetic Association Database for disease and cancer associations. *BCLAF1* deletion was identified as a noteworthy SV that could be associated with OSCC. Transient small interfering RNA-mediated knockdown of *BCLAF1* resulted in the altered expression of several downstream genes, including downregulation of the proapoptotic genes Caspase-3 and BAX and the DNA damage repair genes exonuclease 1, ATR-interacting protein and transcription regulator protein BACH1. *BCLAF1* deficiency also attenuated P53 gene expression. Inhibition of *BCLAF1* expression also resulted in increased colony formation. These results provide evidence that the abrogation of *BCLAF1* expression results in the dysregulation of several cancer signalling pathways and abnormal cell proliferation.

Correspondence to: Professor M. Iqbal Parker, Department of Integrative Biomedical Sciences, Division of Medical Biochemistry and Structural Biology, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, Cape Town, Western Cape 7925, South Africa
E-mail: iqbal.parker@uct.ac.za

Present addresses: [2]Department of Natural and Applied Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Private Bag, Windhoek, Khomas 13388, Namibia; [3]Institute for Glycomics, Griffith University, Gold Coast, Queensland QLD4215, Australia

Abbreviations: ADH4, alcohol dehydrogenase 4; BCLAF1, BCL2 associated transcription factor 1; DDR, DNA damage response; DMEM, Dulbecco's modified Eagle's medium; DSB, double strand break; InDels, insertions and deletions; MSH3, muts homolog 3; OSCC, oesophageal squamous cell carcinoma; SNP, single nucleotide polymorphisms; WGS, Whole Genome Sequencing

Key words: tumour mutations, BCL2 associated transcription factor 1 deletion, insertions and deletions, Whole Genome Sequencing

Introduction

Cancers arise as a result of genetic changes in the genome such that cancer genomes contain inherited and sporadic nucleotide sequence changes. In addition to hereditary predisposition, direct mutational events and/or epigenetic modifications initiate changes in signalling pathways that can result in cellular transformation (1). At a molecular level, cancer development is a multistep process with alterations in the expression of three gene classes; namely oncogenes, tumour suppressor genes and stability genes (2,3). Overexpression of oncogenes and loss of tumour suppressor gene functions result in constitutive activation of pathways under conditions in which the wild-type gene is not active (4,5). By contrast, mutations in stability genes increase genetic instability, resulting in inefficient DNA repair mechanisms and cell cycle control (6,7).

The DNA sequence changes may include insertions and deletions (InDels), gene duplication, genomic rearrangements, loss of heterozygosity in somatic cells or inherited mutations (8). Given that InDels are more likely to be driver gene mutations, a substantial number of InDels in the coding DNA sequence is not surprising, since InDels are often deleterious as evidenced

by their frequent association with human disease (9,10). InDels have also been shown to be components of gene and pseudogene evolution that are important in the long-term evolution of genome size (11). Thus, the abundance of InDels, which have a comparatively greater influence than single nucleotide polymorphisms (SNPs), is consistent with the abnormal function and devastating characteristics of cancer (10).

Our previous Whole Genome Sequencing (WGS) study in patients with oesophageal squamous cell carcinoma (OSCC) revealed large scale deletions of the BCL2 associated transcription factor 1 (*BCLAF1*) gene (12). *BCLAF1* encodes a multifunctional protein with four splice variants that perform a wide range of seemingly unrelated functions (13). Some of these functions include tumour suppression (through promoting apoptosis, senescence, DNA repair and autophagy), embryonic development, T cell maturation and mRNA processing, as illustrated in Fig. 1.

BCLAF1 was originally identified in a yeast protein screen for E1B 19K interacting factors. E1B 19K is a homolog of anti-apoptotic BCL-2 family members and inhibits cell death signal transduction cascades activated by adenoviral proteins (13,14). Overexpression of *BCLAF1* induces apoptosis, suggesting that *BCLAF1* is a pro-apoptotic factor with potential tumour suppressor activity (13). However, the role of *BCLAF1* as a tumour suppressor gene that promotes apoptosis and autophagy is debatable due to conflicting lines of evidence (15,16). Despite this, *BCLAF1* mutations and loss of gene expression have been implicated in a range of cancers, including oesophageal cancer, non-Hodgkin's lymphoma, Burkitt's lymphoma, colon cancer, bladder cancer and rectal cancer (17). Understanding of the genomic abnormalities in OSCC is very limited, hence the need to identify genomic abnormalities underlying OSCC and to elucidate the molecular basis of the disease in order to guide the development of effective targeted therapies (18,19).

Although, *BCLAF1* deletions have been observed in a number of tumour types, very little is known concerning *BCLAF1* expression patterns in human cancers and the effects of its deficiency on gene expression and tumourigenesis (13,20). The present study aimed to identify structural variants (SVs), primarily large InDels, and the downstream molecular mechanisms involved in cellular transformation following the loss of *BCLAF1* gene expression in a cultured cell line model.

Materials and methods

Sample collection. Patients were recruited at Groote Schuur Hospital, University of Cape Town (Cape Town, South Africa) in the gastrointestinal clinic between January 2010 and December 2021. Written informed consent was obtained from all patients before recruitment into the study. Patient ages ranged between 40 and 80 years, and the ratio of female to male patients was 60:40. Tumours were identified by endoscopic examination and biopsy after staining with acetic iodide solution. Normal biopsies were taken at least 10 cm from the tumour. Patients who had previously received any form of chemo- or radiotherapy were excluded from the study (21). Ethics approval was obtained from the University of Cape Town/Groote Schuur Hospital Human Research Ethics Committee (Cape Town, South Africa; approval no. HEC040/2005), and all methods were performed in accordance with the Declaration of Helsinki.

DNA extraction from tissue biopsies. Frozen tissue was thawed on ice and placed into a sterile 1.5 ml microcentrifuge tube containing 0.5 ml QIAzol Lysis Reagent™ (Qiagen, Inc.). The tip of a disposable probe was then placed in the tube and homogenised at full speed until the tissue lysate was uniformly homogeneous (1-5 min). An additional 0.5 ml QIAzol Lysis Reagent was added and mixed by vortexing for 30 sec, after which the samples were incubated for 5 min at room temperature (20-25˚C) to permit the complete dissociation of nucleoprotein complexes. A total of 0.2 ml chloroform per 1 ml QIAzol Lysis Reagent was then added to each tube, capped and shaken vigorously by hand for 15 sec and incubated at room temperature for 2-3 min. The samples were centrifuged at 12,000 x g for 15 min at 4˚C to separate the mixture into three phases: A colourless upper aqueous phase containing RNA, a white middle interphase containing DNA and a lower organic phenol phase containing protein. The upper aqueous phase was transferred to a new tube and stored at 4˚C for later RNA extraction. A total of 0.3 ml 100% ethanol was then added to the interphase and mixed by inversion followed by incubation on ice for 30 min. The samples were then centrifuged at 14,000 x g for 5 min at 4˚C to pellet the DNA, the ethanol was discarded and the pellet was rinsed in 1 ml 75% ethanol. The samples were centrifuged at 14,000 x g for 5 min at 4˚C, and the supernatant was carefully removed and the DNA pellet was air dried for 5-15 min. The DNA pellet was dissolved in 200 μl 10 mM Tris/1 mM EDTA and quantitated on a NanoDrop 2000/2000c Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Samples were stored at -20˚C until required.

Variant calling. Four OSCC DNA biopsy samples were submitted to the Centre for Proteomic and Genomic Research (Cape Town, South Africa) for paired-end sequencing on the Illumina HiSeq2000 (Illumina, Inc.) using TruSeq SBS chemistry v3. or WGS. Quality control (QC) measures were carried out to validate the integrity of the samples for WGS with the Quant-iT™ PicoGreen™ dsDNA kit (cat. no. P7589; Invitrogen; Thermo Fisher Scientific, Inc.). Paired-end sequencing was performed on the Illumina HiSeq2000, with 2x100 bp reads. Reads were aligned to the NCBI37 Homo sapiens reference genome using ELAND and CASAVA version 1.8.2 software (22). Since matched normal samples were not included, variants with global allele frequency >10% in either the Exome Variant Server or 1000 Genomes Project were filtered out to reduce background noise. SVs reported from the alignment were collated with gene loci, (upstream, downstream and overlapping) using the Variant Effect Predictor of Ensembl (23). The samples were genotyped on an Illumina HumanOmni2.5-8 array (Illumina, Inc.) and the affected genes were subsequently cross-checked against the Genetic Association Database for disease and cancer associations (24). The deleted genes common to all the four tumour samples were described in HUGO Gene Nomenclature Committee (HGNC) (25) and Medical Subject Headings (MeSH) gene and disease terms, respectively (Courtesy of the U.S. National Library of Medicine).

WGS. DNA isolated from tumour biopsies obtained from patients with oesophageal squamous cell carcinoma (OSCC) were sent to the Centre for Proteomic and Genomic Research (CPGR), Cape Town, South Africa for WGS. The samples were taken from the Black and Mixed Ancestry population groups since these are the population groups most affected by OSCC in South Africa. The Quant-iT™ PicoGreen™ dsDNA kit (cat. no. P7589; Invitrogen; Thermo Fisher Scientific, Inc.) was used to determine DNA integrity and 10 nM DNA sample was used for sequencing. Paired-end sequencing was performed on the Illumina HiSeq2000, with 2x100 bp reads. PCR-free paired end libraries were manually generated from 500 ng 1 μg genomic DNA using a modified version of TruSeq DNA v2 Sample Preparation kits (cat. no. FC-121-2001; Illumina, Inc.). Fragmentation was performed using Covaris LE220 (Covaris, Inc.). After fragmentation and end repair, libraries were manually size-selected using bead-based size selection targeting 300 bp inserts (Agencourt AMPure XP A63881; Thermo Fisher Scientific, Inc.). Following size selection, libraries were A-tailed, and ligated before proceeding to library QC. Final Libraries were quality checked on a Caliper GX (PerkinElmer, Inc.) and quantified by reverse transcription-quantitative (RT-q)PCR on the Roche LightCycler 480 (Roche Diagnostics). Samples were clustered on three lanes with the Illumina v3 flowcells (Illumina, Inc.) using the Illumina cBot (Illumina, Inc.) with the TruSeq Paired End Cluster Kit (v3). Flowcells were sequenced as 100 bp-end reads on the Illumina HiSeq2000 using TruSeq SBS chemistry v3. DNA sequences were compared to the human reference genome (NCBI37), to perform a preliminary screen for InDels.

Maintenance of cells in culture. CT1 cells were obtained from Dr Namba and cultured as previously described (26) were cultured in complete medium [Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% heat inactivated foetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) and 100 U/ml penicillin and 100 mg/ml streptomycin] at 37˚C in a humidified incubator with 5% CO_2. Every 2 days, the medium was changed and the cells were sub-cultured when they reached 90% confluency, by detaching the cells with 0.05% trypsin-EDTA. Once detached, the trypsin-EDTA was inactivated by the addition of 5 ml complete DMEM. Cells were collected by centrifugation at 3,000 x g at 4˚C for 5 min, suspended in complete DMEM and re-plated at a ratio of 1:3. Cells were regularly checked for mycoplasma contamination as previously described (27).

Small interfering (si)RNA transfection. Transient transfection with siRNA was used to knock down the expression of *BCLAF1*. Briefly, $2x10^5$ cells were plated in a six-well dish (35 mm per well) with complete medium and incubated overnight at 37˚C, in a humidified 5% CO_2 incubator. The following day, the cells were washed once with 2 ml DMEM, then transfected using TransFectin™ Lipid Reagent (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. Two transfection mixtures were prepared separately for each siRNA; the first mixture contained 1.5 μl (75 pmoles) siRNA in 50 μl DMEM and the second mixture had 10 μl TransFectin Lipid Reagent in 45 μl DMEM. After combining the mixtures and incubating them at room temperature for 45 min, the combined mixture was pipetted drop-wise into each well. The plates were incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 5-7 h, after which, 1.750 ml DMEM containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin was added and the cells incubated for an additional 48 h. Untransfected cells and a non-targeting siRNA were used as controls. Each experiment was conducted in triplicate. The siRNA duplex sequences are listed in Table SI.

RNA preparation and RT-qPCR. Total RNA was extracted as previously described using TRIzol® reagent (Thermo Fisher Scientific, Inc.) (27). cDNA was prepared from 2 μg total RNA using the ImProm-II™ Reverse Transcription System (Promega Corporation), according to the manufacturers' instructions. RT-qPCR assays were performed using SYBR® FAST qPCR kit (Roche Diagnostics) in a reaction volume of 20 μl. All amplifications were performed as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C for 20 sec and 72˚C for 5 sec. Analysed genes were amplified in triplicate using the LightCycler® 480II (Roche Diagnostics) and normalized to GAPDH expression in the same sample using the $2^{-\Delta\Delta Cq}$ method (28). A list of the primers used and their annealing temperatures are shown in Table SII.

Western blot analysis. Control cells and cells transfected with siRNA were lysed in RIPA buffer [10 mM Tris-HCl pH 7.6, 10 mM NaCl, 3 mM MgCl2 and 1% (v/v) Nonidet P-40 containing 50 μg/ml each of pepstatin, leupeptin and aprotinin]. Total protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Inc.). Total cell lysates (50 μg protein) were separated by electrophoresis on 12% SDS-PAGE gels under reducing conditions (50 mM β-mercaptoethanol). After electrophoresis, proteins were transferred to nitrocellulose membranes and blocked in 5% fat-free milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 with shaking for 1 h at room temperature. The membranes were incubated overnight at 4˚C with one of the following primary antibodies (1:1,000; all purchased from Abcam): Rabbit polyclonal anti-BCL2 associated transcription factor 1 (BTF; cat. no. ab107177), rabbit polyclonal anti-caspase-3 (phospho S150; cat. no. ab59425), rabbit polyclonal anti-Caspase-9 (cat. no. ab209495), rabbit monoclonal anti-BAX (cat. no. ab182733), rabbit polyclonal anti-Bcl-2 (cat. no. ab115807), rabbit polyclonal anti-Exonuclease 1 (EXO1; cat. no. ab106303), rabbit polyclonal anti-γ H2AX (phospho S139; cat. no. ab11174), rabbit polyclonal anti-p53 (cat. no. ab17990) and rabbit polyclonal anti-p21 (cat. no. ab219811). After several washes in TBS with 0.1% Tween-20 buffer, the nitrocellulose membranes were incubated by shaking at room temperature for 1 h with the required horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3,000; cat. no.1706515; Bio-Rad Laboratories, Inc.) and detected using LumiGLO® substrate (KPL, Inc.).

Soft agar assay. The anchorage dependence and colony formation ability of the cells was assessed using a soft agar assay. Cells ($2x10^5$) were transfected with 75 ρmoles siRNA. Following transfection, cells were washed with 2 ml PBS and

trypsinised with 500 μl 0.5% trypsin-EDTA. Then, 500 μl DMEM was added to inactivate trypsin. The cells were centrifuged at 3,000 x g at 4˚C for 5 min and suspended in 1 ml DMEM. A base agar was prepared by mixing 1% agarose gel at 40˚C with 2X DMEM (20% FBS and 2% P/S). Following which, 1 ml base agar was added to each well of a 6-well plate and gently swirled to cover the surface. The agar was allowed to set for 30 min at room temperature. Top agar was prepared by dissolving 0.7% agarose in a microwave, then cooling it to 40˚C. A suspension of 2,500 cells in 0.1 ml was mixed with 3 ml 0.7% agar and 3 ml 2X DMEM. Then, 1 ml cell suspension was pipetted onto the base agar in triplicate. The cells were incubated at 37˚C in a humidified 5% CO_2 incubator for 21 days. The cells were fed twice a week with 1 ml complete medium. After 21 days, 0.5 ml 0.005% crystal violet (Sigma-Aldrich; Merck KGaA) was added for 1 h and pink stained colonies were counted in 15 different fields using an Olympus CKY41 light microscope (Olympus Corporation) at x100 magnification. ImageJ (version 1.53i) software (National Institutes of Health) was used for semi-quantification of the number of colonies >150 μm in diameter.

Statistical analysis. The data are presented as the mean ± SEM. All experiments were performed in triplicate unless stated otherwise. Statistical analysis of the data was performed using GraphPad Prism 5 (GraphPad Software, Inc.). A two tailed unpaired Student's t-test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SVs in OSCC. WGS data generated 4.27 billion reads of 100 bp covering at least 95% of the human reference genome. QC indicators, such as the duplication rate, coverage and mean depth of coverage, indicated that the experiments were successful in acquiring high-quality sequence reads (Table SIII). The minimum depth of coverage was 33.7, and the library preparation was successful across all samples without being compromised by PCR amplification bias after having obtained a 98% unique read proportion (Non-N reference) to the human reference genome (NCBI37).

The samples were genotyped on an Illumina HumanOmni2.5-8 array, and had a high quality score with a percentage base calling of >Q30 indicating a smaller probability of error. Fig. S1A shows that sample B381 had 89.0% of filtered and aligned reads with a mean depth of coverage of 40.7, while Fig. S1B shows that sample B450 had 90.7% of filtered and aligned reads with a mean depth of coverage of 35.0. Sample M456 had 89.3% of filtered and aligned reads with a mean depth of coverage of 33.6 (Fig. S1C). Whereas, sample M478 had 88.3% of filtered and aligned reads with a mean depth of coverage of 34.2 (Fig. S1D). Thus, the sequencing phase of this experiment achieved high quality reads that allowed for greater confidence in the variant calling procedure and results.

Alignment and variant calling. Illumina's proprietary alignment algorithm ELAND and CASAVA 1.8.2 software were used to align the reads to the NCBI37 human reference genome (22). As matched normal samples were not included,

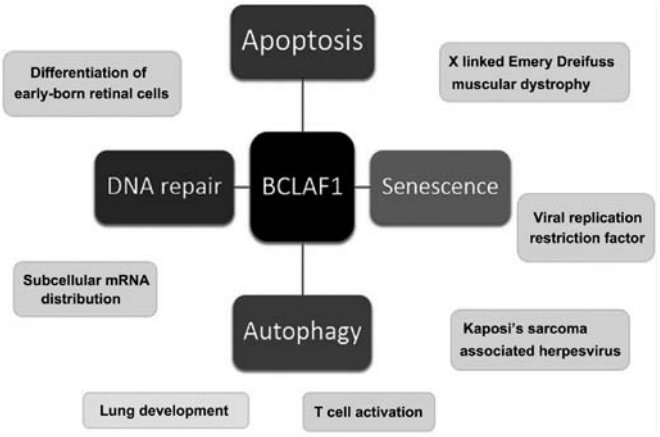

Figure 1. Summary of the currently known functions of *BCLAF1*. BCLAF1 is linked to several cancer-related molecular mechanisms that BCLAF1 has been shown to regulate in multiprotein complexes. The grey (unlinked) boxes are other independent pathways and processes that *BCLAF1* regulates. BCLAF1, BCL2 associated transcription factor 1.

variants with global allele frequency >10% in either the Exome Variant Server or 1000 Genomes Project were filtered out to reduce background noise (29,30). SVs reported from the alignment were collated with gene loci (upstream, downstream and overlapping) using the variant effect predictor of Ensembl (23). The affected genes were subsequently cross-checked against the Genetic Association Database for disease and cancer associations (24). A large number of SNP effects from all four samples were observed (Table I), having 98% mean array agreement between the samples and the reference genome NCBI37 of which 38% were associated with Genes.

The transition/transversion (Ti/Tv) ratio has been used in multiple studies for assessing the specificity of SNP calls, with an empirical Ti/Tv ratio of ~2.1 for genome-wide variants (31-33). Ti/Tv ratio is computed as the number of transition SNPs divided by the number of transversion SNPs (34). Typically, the Ti/Tv ratio is lower in novel SNPs than in known SNPs because of residual false positives and a relative deficit of transitions due to sequencing context bias (31). All the samples in the present study had a Ti/Tv ratio of 2.1, thus further validating the accuracy of SNP calling. The SNP sequence variation in the chromosome showed 90% call accuracy to the reference genome (NCBI37) across all the four samples, which was replicated with the autosomal, mitochondrial and the X-specific sites (data not shown).

The most prevalent SVs were insertions, deletions and copy number variation. Very few insertions were observed in all the four samples compared to deletions and copy number variations, which were common (data not shown). This study concentrated on identifying large SVs (>300 bp) and the role that they could play in OSCC. Since no common insertions were identified in all four samples, the evaluation of insertions was not explored further in this study.

Effects associated with deletions. A total of 697 deletions were observed in all four samples of which 349 within genes and 127 were associated with various cancers (Table II). Only 12 genes were shown to have deletions in the coding region or were associated with OSCC (Table III). Among the

Table I. Summary of SNP consequences across all four tumour samples.

	Tumour samples			
Variant effects	B381	B450	B456	M478
Array agreement, %	98.5	99.2	97.6	99.2
SNP total, n	4,919,446	5,334,173	4,185,808	5,018,129
Genes, %	38.9	38.9	38.8	39.1
Exons, %	1.6	1.6	1.6	1.6
Ti/Tv, n	2.1	2.1	2.1	2.1
Novel, n	591,283	617,474	476,394	656,181
Ti/Tv novel, n	1.5	1.6	1.4	1.7
Synonymous, n	14,266	15,310	11,750	14,562
Missense, n	13,349	14,556	11,243	13,660
Nonsense, n	140	144	118	135
InDel total, n	1,308,522	1,358,218	1,153,901	1,303,928
Frameshift InDel, n	417	431	378	446
Splice region[a], n	1,819	1,892	1,450	1,857

[a],<=5b from splice site; InDels, insertions and deletions; SNPs, single nucleotide polymorphisms; Ti/Tv transition/transversion. Samples were aligned to the NCBI37 *Homo sapiens* reference genome using ELAND and CASAVA, and the SNP consequences reported from the alignment were collated with gene loci using the variant effect predictor of Ensembl. In all four samples there was a 98% mean array agreement between the genotyping and sequencing SNPs to the reference genome NCBI37 of which 38% were associated with Genes and the Ti/Tv ratio was 2.1, further validating the accuracy of SNP calling. The affected genes were subsequently cross-checked against the genetic association database for disease and cancer associations.

Table II. Summary of deletions common to all four tumour samples that shows the breakdown of the total number of deletions described in the samples.

Deletions	Common in all four samples, n
Number of common deletions	697
Deletions associated with genes	349
Deletions associated with cancer	127

Deletions associated with genes were cross-checked against the genetic association database for disease and cancer associations.

twelve genes; alcohol dehydrogenase 4 (*ADH4*), *EGFR*, muts homolog 3 (*MSH3*) and *BCLAF1* have previously been associated with cancer (35,36), but only *BCLAF1* was identified in the present study as a possible noteworthy SV owing to its novel association with OSCC.

The *BCLAF1* deletion had previously been shown to be associated with Non-Hodgkin's lymphoma and colorectal cancer (13,17). In the current study, the *BCLAF1* deletion spanned 53.20% of the protein coding region as opposed to the other SVs and was proposed to have significant implications for the progression of OSCC.

siRNA-mediated BCLAF1 silencing. siRNA-mediated knockdown of *BCLAF1* was determined at both the protein and mRNA levels. A decreased expression of *BCLAF1* expression at both the mRNA and protein levels was observed after transfection with *BCLAF1*-siRNA (Figs. 2A and B and S2A).

Effect of BCLAF1 silencing on cellular gene expression. To assess whether the expression of genes downstream of *BCLAF1* was altered by the targeted disruption of *BCLAF1* expression, RNA and protein extracts from cells transfected with either non-specific scrambled control siRNA, *BCLAF1* siRNA and untreated cells were collected 24 h post-transfection. RT-qPCR and western blot analyses were performed to determine the effects of *BCLAF1* deficiency on pro-apoptotic, anti-apoptotic, DNA damage response (DDR) genes and cell cycle regulators. *BCLAF1* knockdown resulted in a significant decrease in the expression of the apoptotic genes, such as BAX and Caspase-3, gene expression (Figs. 3A-D and S2B-D and Table SIV). Analysis of both the protein and mRNA levels showed no significant effect on the expression of the anti-apoptotic gene BCL2, but a significant effect was observed on the expression of cell cycle regulators such as P53 (Figs. 4A-D and S2E and H and Table SIV). Depletion of *BCLAF1* also resulted in reduced expression levels of EXO1, ATRIP and BACH1 mRNA and the slight attenuation of EXO1 and H2AX protein levels (Figs. 5A-F and S2F and G and Table SIV).

Effect of BCLAF1 silencing on anchorage-dependent growth. To determine the effect of *BCLAF1* on anchorage-dependent growth and cellular transformation, soft agar colony forming assays were performed. Normal cells are anchorage dependent and undergo apoptosis in the absence of a binding substrate. Transformed cells on the other hand, proliferate to form colonies (27). After incubation for 21 days, colonies were stained

Table III. Description of gene deletions common to all four tumour samples.

Genes (HGNC)	Location	Variant consequence	MeSH disease terms	Percentage protein coding overlap, %
NXPE1	Chr.11	Non-coding exon variant	Ulcerative colitis; inflammatory bowel disease	0.66
DLEU1	Chr.13	Non-coding exon variant	Chronic lymphocytic leukaemia	0.32
TMBIM4	Chr.12	3'UTR variant	Venezuelan haemorrhagic fever; Bolivian haemorrhagic fever	0.73
ADH4	Chr.4	Upstream gene variant	Adenocarcinoma; oesophageal neoplasm; Parkinson's disease	0.12
HYDIN	Chr.16	Coding sequence variant	Primary ciliary dyskinesia 5; Hydin-related primary ciliary dyskinesia	0.49
KCNJ12	Chr.17	Frameshift variant 3'UTR	Hypokalemic periodic paralysis	3.53
BCLAF1	Chr.6	Coding sequence variant	Non-Hodgkin's lymphoma; colorectal cancer	53.20
CACNA1B	Chr.9	Coding sequence variant	Pulmonary aspergilloma	0.34
EGFR	Chr.7	Intron variant	Oesophageal neoplasms; colonic neoplasms; cervical squamous cell carcinoma	0.24
FAM118A	Chr.22	Coding sequence variant	Cardiovascular diseases; Diabetes Mellitus, Type 2	1.70
OR6K5P	Chr.1	Non-coding exon variant	Neuronitis	25.35
MSH3	Chr.5	Intron variant	Adenocarcinoma; oesophageal neoplasm; colonic neoplasms	0.31

HGNC, HUGO Gene Nomenclature Committee; MeSH, Medical Subject Headings. Percentage overlap of the protein coding region was reported from the alignment to the human reference genome (NCBI37), and collated with gene loci using the Variant Effect Predictor of Ensembl.

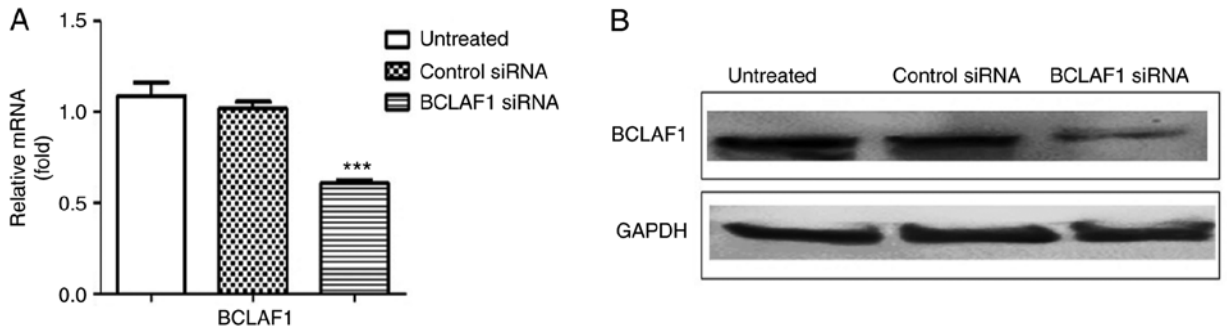

Figure 2. Effect of *BCLAF1* knockdown on protein and mRNA expression. CT1 cells were seeded in 6-well plates ($2x10^5$ cells per well) and transfected with either the control siRNA or *BCLAF1* siRNA (75 pmoles). (A) Reverse transcription-quantitative PCR analysis of *BCLAF1* mRNA levels. (B) Representative western blot analysis of proteins extracted from the control and transfected cells. All blots were from the same gel. Separate amplification of GAPDH served as a housekeeping gene to control for loading. Each experiment was performed in triplicate. ***P<0.001 vs. control siRNA. BCLAF1, BCL2 associated transcription factor 1; siRNA, small interfering RNA.

with 0.005% crystal violet, visualised and counted. There was a notable increase in size and number of colonies following *BCLAF1* silencing compared with the controls (Fig. 6A-D).

Discussion

Cancer-derived cell lines are generally used as models in the study of cancer, but there is no perfect cell line model due to a number of well-known limitations (37-42). Although cancer research is commonly conducted in epithelial cells, tumours are heterogeneous and the tumour microenvironment aids carcinogenesis (43). Due to the ability of immortalized cells to surpass replicative barriers in culture, they constitute a robust model system that can be used to study human cell transformation (43,44). CT1 fibroblasts were used as a model cell line in the present study to validate the effects of BCLAF1 knockdown on cellular transformation. The CT1 cells used in this study did not contain any p53 mutations and were thus suitable for studying the relationship between cell immortalization and changes in p53 gene expression (26,45) as opposed to human cell lines that are immortalized by treatment with oncogenic DNA viruses, such as SV40, papilloma and adenoviruses, since these viruses bind to and inactivate the proteins, such as p53 and Rb (46,47).

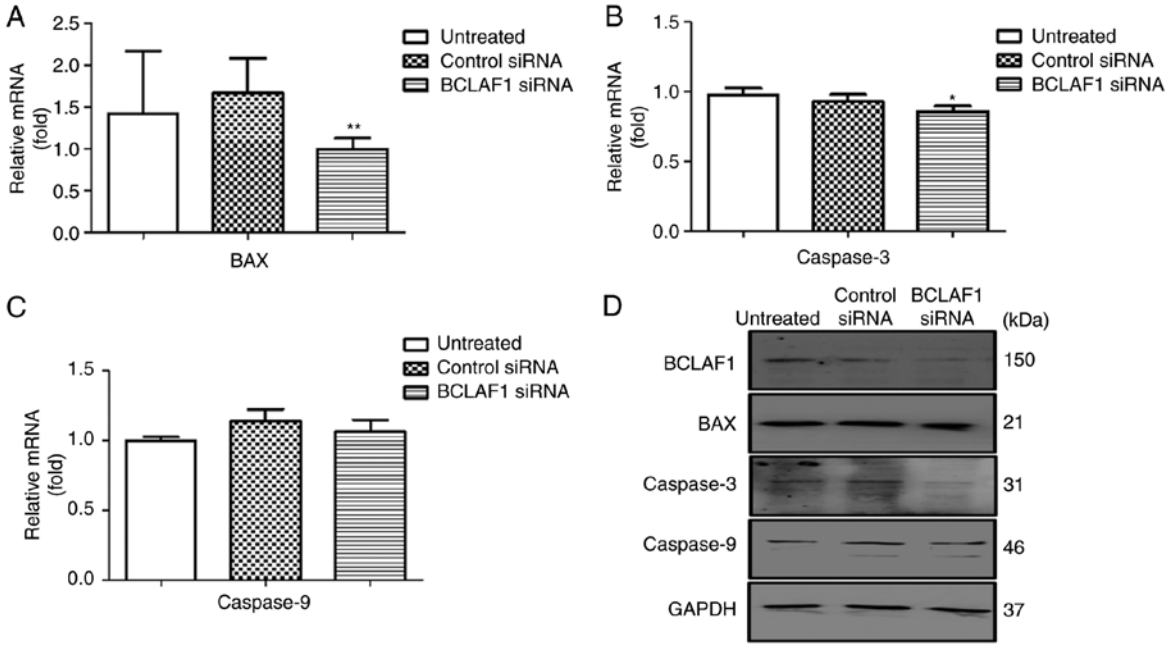

Figure 3. Effect of *BCLAF1* knockdown on proapoptotic gene expression. Cells were transfected with either control siRNA or *BCLAF1* siRNA, and RNA was extracted for RT-qPCR. (A-C) RT-qPCR analysis of the expression of the pro-apoptotic genes BAX, Caspase-3 and Caspase-9 mRNA levels using specific primers. (D) Representative western blot analysis of BAX, Caspase-3 and Caspase-9 proteins. Separate amplification of GAPDH served as a housekeeping gene to control for loading. Each experiment was performed in triplicate. *P<0.05, **P<0.01 vs. control siRNA. BCLAF1, BCL2 associated transcription factor 1; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR.

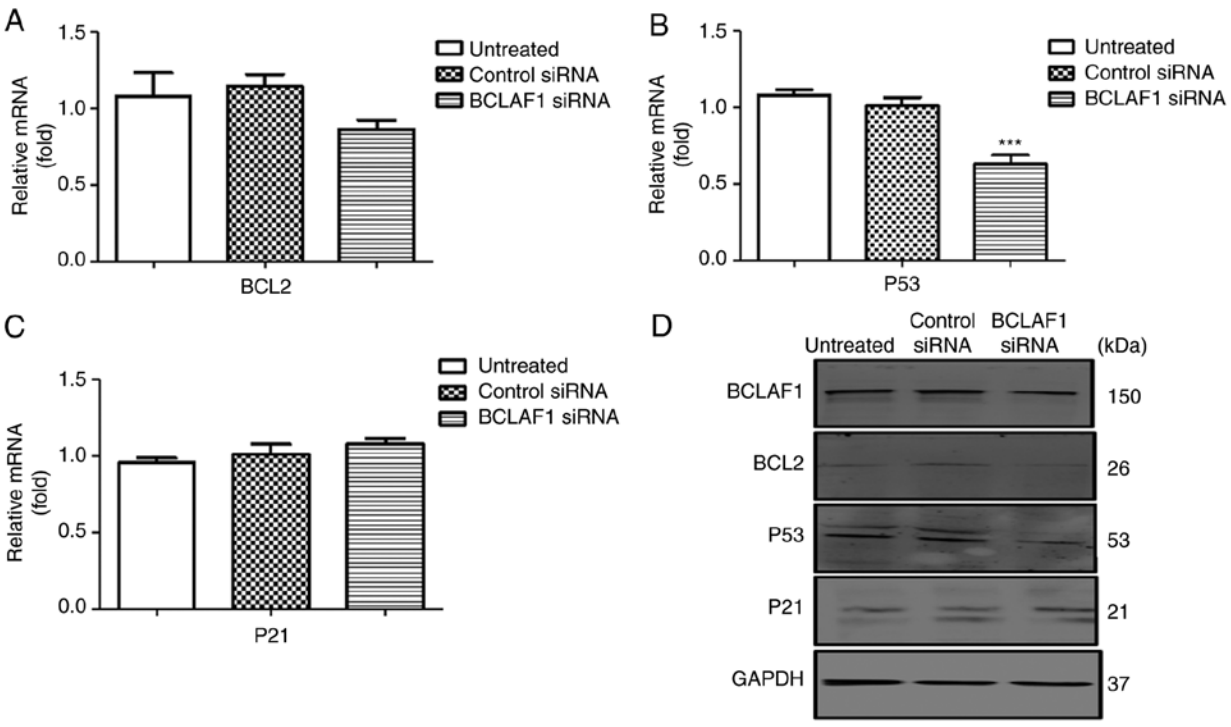

Figure 4. Effect of *BCLAF1* knockdown on anti-apoptotic genes and cell cycle regulators. Cells were transfected with either control siRNA or *BCLAF1* siRNA, and RNA was extracted for RT-qPCR. (A-C) RT-qPCR analysis of the anti-apoptotic gene BCL2 and cell cycle regulators P53 and P21. (D) Western blot analysis of BCL2, P53 and P21 protein levels. Double bands observed in some lanes (P21 and P53) were likely due to protein modifications such as phosphorylation. Each experiment was performed in triplicate. ***P<0.001 vs. control siRNA. BCLAF1, BCL2 associated transcription factor 1; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR.

In the present study, four OSCC samples were subjected to WGS to identify large SVs (InDels) that may be associated with OSCC. A total of 4.27 billion reads of 300 bp fragments were generated covering 95% of the human reference genome (NCBI37) at a mean read depth of 33.7. This allowed for the reliable calling of SNPs and SVs across the human reference

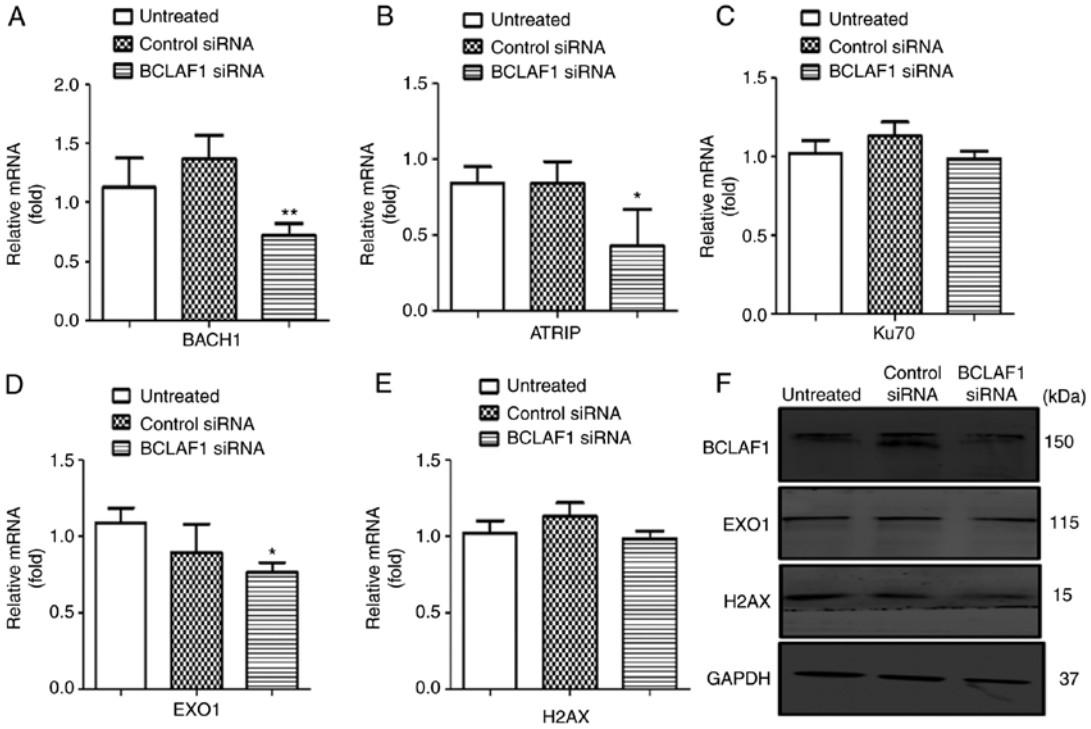

Figure 5. Effect of *BCLAF1* knockdown on double strand DNA repair gene expression. (A-E) Reverse transcription-quantitative PCR analysis of the DDR genes BACH1, ATRIP, Ku70, H2AX and EXO1. (F) Representative western blot analysis of DDR proteins, including BCLAF1, EXO1 and H2AX in *BCLAF1* siRNA-treated cells. Separate amplification of GAPDH served as a housekeeping gene to control for loading. Each experiment was performed in triplicate. *P<0.05, **P<0.01 vs. control siRNA. DDR, DNA damage response; BCLAF1, BCL2 associated transcription factor 1; siRNA, small interfering RNA; BACH1, transcription regulator protein BACH1; ATRIP, ATR-interacting protein; EXO1, exonuclease 1; Ku70, 70 kDa subunit of Ku antigen.

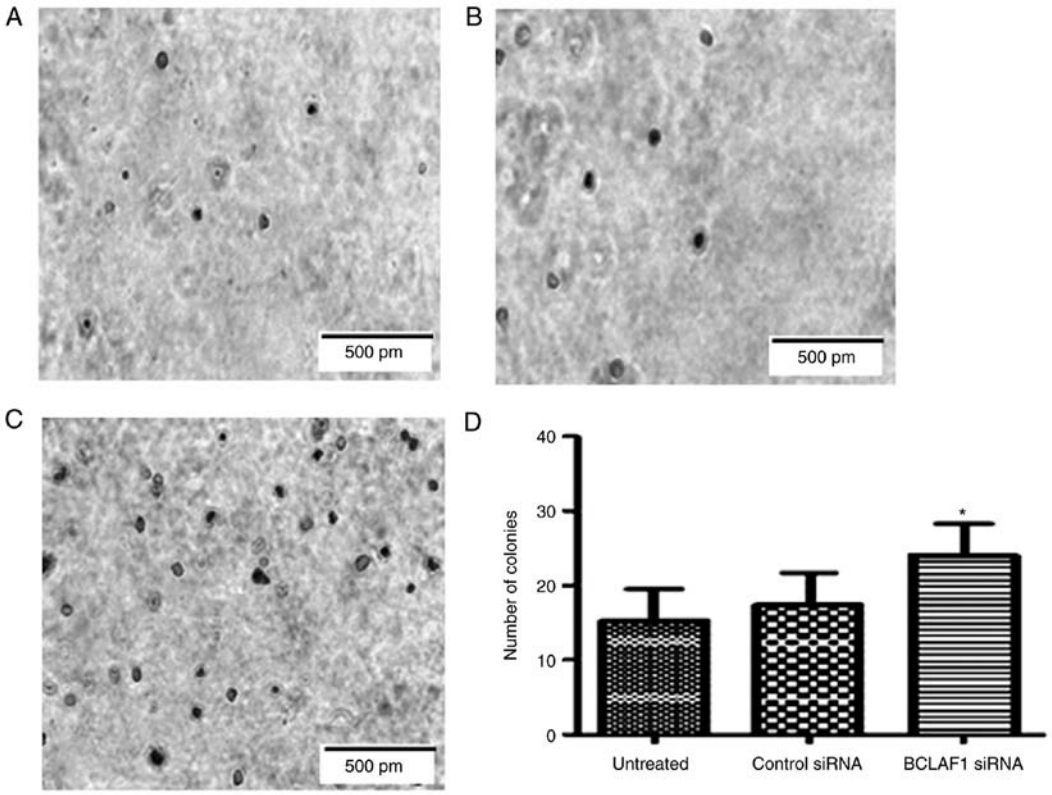

Figure 6. Anchorage-dependent growth assessed by a soft agar assay. CT1 cells were seeded at 2,500 cells/well in 6-well plates with 1% base agar. Images of the number of colonies in the (A) untreated, (B) control siRNA (C) and *BCLAF1* siRNA groups. (D) Graphic representation of the average number of colonies. Cells were plated at a density of $2.5x10^3$ cells/well and colony formation was determined after 21 days. Analysis of the colonies was performed using ImageJ software, with colonies >150 μm in diameter being scored as positive. Scale bar, 500 pm. Graph shows the mean number of colonies ± SEM obtained in three independent experiments, with each experiment performed in six replicate wells. *P<0.05 vs. control siRNA. BCLAF1, BCL2 associated transcription factor 1; siRNA, small interfering RNA.

genome (NCBI37) and agreed with previous studies that also showed that an average read depth that exceeded 30X was the *de facto* standard for achieving reliable variant calling across 95% of the reference genome (48,49).

The Ti/Tv ratio is also a critical metric for assessing the specificity of SNP calling (41) and has been used as a quality control parameter for assessing the overall SNP quality in multiple studies with the expected Ti/Tv ratios in WGS being 2.1, generally indicating higher accuracy in variant calling (31,33). The present results showed a Ti/Tv ratio of 2.1 across all four samples, further confirming the reliability of these SNPs and SV calling.

SVs in the human genome play an important role in the genetics of complex diseases (1,48). Although the analysis of the present study reduced the number of variants of specific interest from the pool of SVs to a select few, large numbers of potentially functional alterations that could contribute to OSCC susceptibility were not investigated. From the 12 potential genes that were common in all four samples, only three genes have been previously shown to be associated with OSCC; *ADH4*, *EGFR*, *MSH3* (17,34,35,50-52) and one novel gene *BCLAF1* that has not been reported in OSCC to date.

A reduction in *ADH4* expression in poorly differentiated OSCC contributes to the maintenance of the tumour state by inhibiting the retinoic acid signalling pathway (53), while mutations in *EGFR* have also been shown to occur in primary OSCC (54,55). Other studies by Vogelsang *et al* (36,56) also showed that *MSH3* polymorphisms, together with exposure to tobacco smoking increased the risk of oesophageal cancer. Although these genes (*ADH4*, *EGFR* and *MSH3*) had previously been associated with OSCC, we did not analyse them further for functional significance in the current study.

The *BCLAF1* deletion on the other hand, is a notable SV due to its novel association with OSCC. Although the exact molecular role of *BCLAF1* remains to be elucidated, *BCLAF1* has been shown to have functional connections and mutation patterns consistent with the known hallmarks of cancer (20,57) suggesting *BCLAF1* as a contributor to the progression of OSCC. Although there are currently no extensive studies depicting the role of BCLAF1 deletion in OSCC, previous studies have shown a role for BCLAF1 deletion in a number of cancers other than OSCC. Ungerbäck *et al* (51) identified BCLAF1 deletion as among the tumour suppressor candidate genes that may be responsible for or a contributing factor to colorectal cancer. Other cancers also linked with the loss of BCLAF1 include hepatocellular carcinoma (58), leukaemia (59), lung cancer (60), bladder cancer (15) and colon cancer (20,61).

BCLAF1 has also been shown to directly interact with and activate *P53* gene transcription and that silencing of *BCLAF1* reduces *P53*-dependent apoptosis, thus implicating *P53* as a downstream target of *BCLAF1* (62). This is also supported by other studies that identified *P53* as among the significantly mutated genes in OSCC (63,64). DDR pathways protect cells from extensive DNA damage and cellular transformation (51,57,65). The network of DDR pathways consists of homologous recombination, mismatch repair, non-homologous end joining and double strand break repair (66). Since there is redundancy in these repair mechanisms and the genes are expressed at low levels in the absence of external DNA damaging agents (5,67), the expression levels of genes that are essential to these processes, including H2AX, BACH1, Ku70, EXO1 and ATRIP, were investigated.

BCLAF1 silencing reduced ATRIP expression in the present study, this was expected due to decreased *BCLAF1*/*BRCA1* complex formation (65). *BCLAF1* silencing had no significant impact on EXO1 expression, but then transcription factors such as MYC and E2F1 also positively regulate the EXO1 promoter (68). Additionally, the RAS/PI3K/AKT signalling pathways induce EXO1 gene expression (69). The activation of these pathways in the transformed cells could compensate for the decreased *BCLAF1* levels. Thus, the *BCLAF1* knockdown was insufficient to cause a significant change in EXO1 expression.

The siRNA-mediated silencing of *BCLAF1* also altered the expression of important DNA damage response regulators, such as ATRIP, H2AX and EXO1. In the event of replicative stress, cells activate several genes to halt cell cycle progression and proliferation. P53 and P21 are potent inhibitors of G1/S phase progression through their interaction with CDK2 and CDK4/6 (70,71). In the G1 phase cell cycle arrest, P53 triggers transcription of P21 (cip1/waf1) a key component in cell cycle regulation (72). The present study confirmed that silencing *BCLAF1* significantly attenuated P53 expression, which was in agreement with the results on *BCLAF1* deficiency and downregulation of Caspase-3 and BAX protein levels.

Caspases regulate the intrinsic and extrinsic apoptosis pathways, resulting in DNA fragmentation, cytoskeletal breakdown and protein degradation (73,74). This current study showed that the expression of Caspase 3 was decreased after knockdown of *BCLAF1* expression. This is likely mediated via the resulting increase in anti-apoptotic Ku70-Bax complexes that causes inhibition of Caspase-3 expression and activation (16). Thus, apoptosis resistance and cell survival are enhanced in si*BCLAF1* treated cells. The BCL-2 family of genes are essential in apoptosis regulation and are either pro-apoptotic (Bax, Bad, Bak) or anti-apoptotic (BCL-2, BCL-XL) (75). The specific underlying mechanisms regarding the induction of apoptosis by *BCLAF1* repression in transformed cells is still largely unknown. Although the transformed cells undergo apoptotic cell death following si*BCLAF1* treatment, they still formed colonies on soft agar.

Although the present study showed that the abrogation of BCLAF1 expression in CT1 fibroblasts resulted in the disruption of several signalling pathways, the fact that a fibroblast cell line rather than an oesophageal cell line for the functional analysis is a limitation in this study.

The current study demonstrated that BCLAF1 deletion may be an important determinant in the progression of OSCC. To the best of our knowledge this is the first study linking BCLAF1 deletion in OSCC. It is possible that the link between BCLAF1 deletion and the progression of OSCC is via the downregulation of P53 and the deregulation of cellular processes involved in carcinogenesis. This hypothesis is also supported by studies that showed the role of BCLAF1 as a tumour suppressor and to promote the stability of genes required for DNA repair and maintenance of genomic stability (13,65).

To conclude, this study contributes towards our understanding of the underlying mechanisms involved in genomic alterations. It also provides evidence that abrogation of

BCLAF1 expression results in the dysregulation of several cancer signalling pathways leading to cellular transformation. This in turn presents an opportunity for the design of novel therapeutic protocols based on the genomic traits of tumours.

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Availability of data and materials

All data generated or analysed during the current study are available in the NLM NCBI dbVar repository (accession no. nstd195; https://www.ncbi.nlm.nih.gov/dbvar/studies/nstd195/).

Authors' contributions

LMM and MIP conceived and designed the experiments. LMM, VC and HS performed the experiments. LM, VC, HS and MIP analysed the data. MIP contributed reagents, materials and analysis tools. LMM and MIP wrote the manuscript. LMM and MIP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the University of Cape Town/Groote Schuur Hospital Human Research Ethics Committee (Cape Town, South Africa; approval no. HEC040/2005), and all methods were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before recruitment into the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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