Downstream Targets of FOXM1: CEP55 and HELLS are Cancer Progression Markers of Head and Neck Squamous Cell Carcinoma

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Short Title: CEP55 and HELLS are biomarkers for HNSCC progression

Keywords

Oral carcinoma, FOXM1, CEP55, HELLS, LSH, PASG, SMARCA6, head and neck, oncogene, diagnostic biomarkers, HNSCC, oral cancer, digital densitometry, early detection, bioinformatics, microarray, immunohistochemistry, qPCR, real-time quantitative PCR.

Abbreviations

HNSCC, head and neck squamous cell carcinoma; FOXM1, forkhead box M1; CEP55, centrosomal protein 55; HELLS, lymphoid specific helicase; qPCR, absolute real time reverse transcription quantitative polymerase chain reaction; NHOK, primary normal human oral keratinocytes; LnMet, lymph node metastasis; FFPE, formalin-fixed paraffin embedded.

ABSTRACT

We recently showed that upregulation of a key oncogene FOXM1 precedes head and neck squamous cell carcinoma (HNSCC) malignancy. Furthermore, we also identified a centrosomal protein CEP55 and a DNA helicase/putative stem cell marker HELLS, which are both downstream targets of FOXM1. In this study, we have investigated the expression profiles of CEP55 and HELLS using immunohistochemistry and quantified by digital densitometry in a tissue panel (20 samples) consisting of normal oral mucosa, dysplasias, HNSCC and lymph node metastasis (LnMet) samples. Furthermore, we corroborated our findings using absolute real-time PCR (qPCR) on a panel of 12 primary normal human oral keratinocytes, 5 dysplasia and 10 HNSCC cell lines. Finally, we validated our study using bioinformatics microarray analysis on an independent HNSCC patient cohort (4 normal and 16 tumours). In normal oral mucosa, CEP55 protein was detected at very low level within the upper differentiated layers. In contrast, CEP55 was highly expressed in oral dysplasia whereas only moderate expression was detected in HNSCC and LnMet. Low level of HELLS expression was detected in the basal cell layer of the normal oral mucosa, moderate level was seen in dysplasia and high levels in both HNSCC and LnMet. These expression patterns were consistent with both qPCR data from the cell line panel and microarray data analysis of TNM-stage defined HNSCC samples confirming the progressive expression pattern of CEP55 and HELLS. To our knowledge, this is the first pilot study demonstrating that both CEP55 and HELLS mRNA and protein expression positively correlate with pre-malignancy and HNSCC progression. This study provides strong evidence that CEP55 and HELLS may be used in conjunction with FOXM1 as a biomarker set for early cancer detection and indicators of malignant conversion and progression.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide and it has higher death rates than cancers of the colon, breast, vulva or melanoma.¹ In the UK, over 4700 new cases are reported annually. Its impact on health services, the patients, their families and society in general is financially crippling because of the cost of treatment and the effect on the patients' cosmetic appearance, speech and eating. In recent years, there has been evidence of an increasing rate of HNSCC incidence in several European countries probably due to the changing trends of alcohol and tobacco consumption and exposure, especially in females.² Because of late tumour detection coupled with high recurrence rate after treatment, the 5-year survival of patients remains poor (average 50%, depending on clinical stage at diagnosis).¹ This supports the need for better screening and diagnostic interventions to improve early detection of HNSCC.

The human transcription factor *FOXM1* gene consists of 10 exons located on the 12p13.3 chromosomal band (telomeric position). Two exons, named exons Va and VIIa, also referred to as exon A1 (or rat exon 6) and A2 respectively, are alternatively spliced.^{3, 4} FOXM1 has a role in cell cycle, DNA repair and maintenance of genomic stability.⁵ We were the first to establish a link between FOXM1 and oncogenesis when we showed that FOXM1 was upregulated in basal cell carcinoma,³ the most common type of cancer in Caucasians worldwide. Subsequent studies of FOXM1 in many different solid human cancer types confirmed its pivotal role in oncogenesis.^{5, 6} We have recently reported that transcriptional upregulation of *FOXM1* precedes malignancy in a number of solid human cancer types including oral, oesophagus, lung, breast, kidney, bladder and uterus.⁷ It has been shown that FOXM1 interacts with the human papilloma virus (HPV) 16 E7 protein⁸ and that HPV16 E6/E7 has been implicated in HNSCC.⁹ It is likely that HPV may play a role in HPV-positive HNSCC through FOXM1 activation. However, it has been shown that the frequency of HNSCCs that have integrated HPV and express high levels E7 is only in the region of 4-16%.^{10, 11}

We have recently shown that aberrant upregulation of FOXM1 serves as a first oncogenic hit by destabilising the genome and subsequent activation of downstream targets which regulates cytokinesis (CEP55, centrosomal protein 55kDa)¹²⁻¹⁴ and epigenetic/stem-cell factor re-programming (HELLS, helicase lymphoid specific)¹⁵⁻¹⁸ leading to malignant transformation (anchorage-independent growth) of a premalignant oral keratinocyte line (SVpgC2a).⁷ However, the protein expression profiles of CEP55 and HELLS during HNSCC progression have not been described. In this study, we have characterised the expression patterns of CEP55 and HELLS using immunohistochemistry and digital densitometry to quantify their protein levels in an HNSCC progression tissue panel consisting of normal oral mucosa, dysplasias, HNSCC and lymph node metastasis (LnMet). We have complemented these findings using absolute real-time reverse transcription PCR (qPCR) on a panel of primary normal human oral keratinocytes, dysplasia and HNSCC cell lines. The data suggest that CEP55 and HELLS in combination with FOXM1 are early markers of cancer detection and they are also indicators of malignant conversion and progression.

MATERIALS & METHODS

Clinical Samples

The use of human tissue was approved by the relevant Research Ethics Committees at each institution. The present study involved 20 patients' formalin-fixed paraffin embedded (FFPE) archival tissues as previously described.⁷

Immunohistochemistry and digital pixel densitometry

FOXM1 was immunostained with a rabbit polyclonal antibody (K-19; Santa Cruz Biotechnology, California, USA) and mouse monoclonal antibodies for both CEP55 (AB57537-100; Abcam plc, Cambridge, UK) and HELLS (anti-LSH H-4 SC-46665; Santa Cruz) on paraffin sections as described.^{3, 7, 19} The reaction product was visualised with diaminobenzidine as a chromogenic

substrate in FFPE tissue sections. As described previously,^{7, 20, 21} digital pixel densitometry was performed using Adobe Photoshop CS3 (Adobe Systems Incorporated, USA) utilising the Colour Range tool for colour-based quantification or Lasso tool for manual selection of epithelial mucosa area and subsequent pixel area readout from the Histogram data chart. Whilst we have also used the ImageJ program for pixel densitometry and obtained similar results, Photoshop was preferred for its accuracy, reproducibility and ease of use.

Cell culture

Primary normal human oral keratinocytes (12 different NHOK primary cultures) were cultured in a modified version of Keratinocyte SFM (K-SFM, #17005-034 Gibco, Invitrogen) medium²² containing 0.09 mM CaCl₂. K-SFM was supplemented with 25 µg/ml bovine pituitary extract, 0.2 ng/ml EGF (#37000-015 Gibco, Invitrogen), and 1% penicillin/streptomycin. Oral premalignant cell lines: OKF6/T,²³ POE9n,²² DOK,²⁴ D19,⁷ D20,⁷ and primary HNSCC derived cell lines: SCC9,²⁵ SCC15,²⁵ SCC25,²⁵ SqCC/Y1,²⁶ UK1,²⁷ VB6,²⁷ CaLH2,²⁷ CaDec12,²⁷ 5PT,²⁷ H357²⁷ were cultured as described previously.⁷

Real-time absolute quantitative RT-PCR

Poly-A+ mRNA extraction, reverse transcription and real-time absolute quantitative PCR (qPCR) protocols are MIQE compliant²⁸ and were performed as described previously⁷ using a LightCycler LC480 instrument (Roche Diagnostic). Primer sequences for *FOXM1B*, *CEP55*, *HELLS* and two stable reference genes (*POLR2A* and *YAP1*) were described previously.⁷ Statistical analysis was performed using the GraphPad InStat software (V2.04a, GraphPad Software, San Diego, CA) for Student's t-test analysis.

Bioinformatics analyses of microarray data

FOXM1, *CEP55* and *HELLS* endogenous mRNA levels were analysed using published microarray data in a study using normal oral mucosa and a panel of TNM-staged defined HNSCC tissues²⁹. The microarray dataset (GDS1584) was obtained from the NCBI's Gene Expression Omnibus (GEO)

database. The Affymetrix gene reference identification for *FOXM1*, *CEP55* and *HELLS* are 202580_x_at, 218542_at and 220085_at, respectively. Gene expression levels were statistically analysed using Student's t-test in Microsoft Excel software.

RESULTS

Protein Expression patterns of CEP55 and HELLS during HNSCC progression

In order to validate our previous finding that CEP55 and HELLS are targets of FOXM1, we investigated the CEP55 (Fig. 1) and HELLS (Fig. 2) protein expression levels by immunohistochemistry on a panel of 5 normal human oral mucosa, 5 oral dysplasia, 5 primary HNSCC and 5 LnMet FFPE archival tissues. In normal oral mucosa, very low level of CEP55 immunostaining was detected in the middle/upper suprabasal layers (Fig. 1A,B - Normal). As the staining was restricted only to the keratinised layers we believe that this may be non-specific staining. In contrast, intense but heterogeneous nuclear and cytoplasmic CEP55 expression was detected in oral dysplasia within the basaloid suprabasal layers (Fig. 1A,B – Dysplasia). Moderately upregulated level of cytoplasmic CEP55 protein was detected in HNSCC tissues (Fig. 1A,B – HNSCC). In LnMet, CEP55 exhibits a heterogeneous expression pattern with higher cytoplasmic and nuclear protein levels detected along the periphery of tumour mass (Fig. 1A,B - LnMet). Digital densitometry was used to quantify the levels of CEP55 expression where 6-10 digital images were captured and quantified from each tissue specimen. Individual data points for each tissue specimen were plotted as a scatter plot (Fig. 1C) and Box-and-Whisker plot (Fig. 1D) to illustrate the distribution pattern of CEP55 protein expression across the tissue panel. Mean $\pm$ SEM analysis plot (Fig. 1E) showed that CEP55 was significantly upregulated in dysplasia and HNSCC. The upregulation of CEP55 in LnMet was not significant due to tissue heterogeneity.

In normal oral mucosa, HELLS nuclear immunostaining was detected strictly within the basal layer of the oral mucosa epithelium (Fig. 2A,B - Normal). In oral dysplasia, HELLS nuclear expression was moderately upregulated and found throughout the basaloid layers (Fig. 2A,B – Dysplasia). High level of nuclear HELLS protein was detected in HNSCC tumour masses (Fig. 2A,B – HNSCC). In LnMet, HELLS exhibits a heterogeneous nuclear expression pattern (Fig. 2A,B - LnMet). Individual data points for each tissue specimen were plotted as a scatter plot (Fig. 2C) and Box-and-Whisker plot (Fig. 2D) as described above for CEP55 expression. The mean expression $\pm$ SEM analysis plot (Fig. 2E) showed that HELLS was significantly upregulated in all three disease tissues with a progressive trend.

Correlation of CEP55 and HELLS mRNA expression with FOXM1

It has been previously established that FOXM1B shows a progressive expression pattern during HNSCC progression.⁷ Since CEP55 and HELLS are downstream targets of FOXM1B, we hypothesise that their expression patterns should show a positive correlation with FOXM1B. To test this hypothesis, we measured the mRNA expression levels of *FOXM1B*, *CEP55* and *HELLS* in a panel of 12 primary normal human oral keratinocytes, 5 dysplasia (OKF6/T, POE9n, DOK, D19 and D20) and 10 primary HNSCC derived keratinocyte cell lines (SCC9, SCC15, SCC25, SqCC/Y1, UK1, VB6, CaLH2, CaDec12, 5PT and H357) using absolute qPCR. Both *CEP55* (Fig. 3A) and *HELLS* (Fig. 3B) showed good expression correlation with *FOXM1B* across the panel of cell lines used.

Bioinformatics microarray analysis of CEP55 and HELLS expression

To further validate the above findings, we performed bioinformatics analysis on an independent HNSCC patient cohort comparing 4 normal oral mucosa and 16 TNM-stage defined HNSCC samples.²⁹ The gene expression data of *CEP55* and *HELLS* were extracted from the microarray dataset (GDS1584) using the NCBI's Gene Expression Omnibus online research tool as described previously.⁷ The expression patterns of *FOXM1*, *CEP55* and *HELLS* all showed similar bell-shaped

profile across the different TNM tumour stages (Fig. 1C) with peak expression at early-mid tumour stages (T1N2bM0 to T4N2bM0) and decrease expression found in more advance tumour stages (T4N2M0 and T4N3M0). Statistical analysis showed that *FOXM1* and *CEP55* were significantly upregulated in the HNSCC (n=16) compared to normal tissues (n=4) (Fig. 3D). The upregulation of *HELLS* expression in HNSCC was not statistically significant due to a greater heterogeneous gene expression within the different HNSCC tumour stages.

DISCUSSION

It is now well established that *FOXM1* is a major oncogene in myriad of human cancers including HNSCC.^{7, 30} In our previous study, we identified *CEP55* and *HELLS* as two downstream targets of *FOXM1*.⁷ Herein, we have provided the first protein expression evidence that both *CEP55* and *HELLS* are differentially expressed during tumour progression from dysplasia to HNSCC and LnMet in human oral tissues. Both their expression patterns correlated well with that of *FOXM1* expression profile across a cell line panel containing normal oral keratinocytes, dysplasia and HNSCC derived cells. The progressive pattern of gene expression of *CEP55* and *HELLS* was further validated using bioinformatics microarray data analysis on an independent cohort of HNSCC study.

CEP55 is a microtubule-bundling protein that localizes to the mitotic spindle during prometaphase and metaphase and to the spindle midzone and the midbody during anaphase and is required for the completion of cytokinesis.¹²⁻¹⁴ Consistent with *CEP55* being a direct transcriptional target of *FOXM1*,⁷ both *CEP55*¹² and *FOXM1*³¹ share organ-specific expression in the testis and thymus of normal adult humans. *CEP55* is upregulated in hepatocellular carcinoma and its overexpression induces anchorage-independent growth, enhanced cell growth at low serum levels and induction of tumourigenesis in nude mice.³² Another study showed that *CEP55* was overexpressed in lung cancer

and is associated with cell migration and invasion.³³ Consistent with a role in malignancy, similar to the expression of FOXM1,⁷ we found that CEP55 expression is significantly upregulated in both oral dysplasia and HNSCC tissues. Interestingly, a study involving 3 independent breast cancer patient cohorts of microarray datasets containing a total of 699 patients revealed that CEP55 and FOXM1 are amongst the signature prognostic markers which predict poor breast cancer outcome.³⁴ Another study showed that FOXM1 and CEP55 were ranked 3rd and 43rd, respectively, most significant predictor genes for poor clinical outcome in 6 different cancer types.³⁵ Consistent with being a potential cancer biomarker, CEP55 has been shown to act as a novel breast carcinoma-associated antigen³⁶ which has important implications in cancer diagnostic and/or therapeutic interventions.

Whilst we have previously demonstrated using chromatin-immunoprecipitation that FOXM1 protein directly transactivated the promoter of CEP55 to induce gene expression, we find no evidence of a FOXM1-binding site on the HELLS promoter, despite showing a strong correlation in gene expression between FOXM1 and HELLS.⁷ Our current data also showed significant correlation in both protein and mRNA expression pattern between HELLS and FOXM1 during HNSCC progression. Similar to FOXM1³¹ and CEP55,¹² HELLS also showed organ-specific expression in the testis and thymus.³⁷ Nevertheless, because only 1kb of the HELLS promoter region was analysed,⁷ the FOXM1 binding site(s) could be located beyond the HELLS promoter region tested. Further study involving a larger promoter region of HELLS is required to establish the basis for HELLS induction by FOXM1.

HELLS is a member of the SNF2 (Sucrose Non-Fermenter) family of helicase proteins implicated in mammalian development and cellular proliferation,^{16, 38} through chromatin-remodelling and DNA methylation.³⁹⁻⁴² Whilst deletion of HELLS gene causes developmental retardation, premature ageing and cellular senescence,^{38, 43-45} gene modification or upregulation of HELLS has been implicated in various human cancer including leukaemia,³⁷ non-small cell lung cancer,⁴⁶ breast cancer,⁴⁷ and melanoma.⁴⁸ HELLS was recently identified as one of the consensus genes expressed in human embryonic stem cells.¹⁷ Recently, DNA methyltransferase 1 (DNMT1), an interacting partner of

HELLS,⁴² was shown to maintain progenitor function in self-renewing epidermis⁴⁹ highlighting a role for HELLS in progenitor cell maintenance and renewal. Interestingly, in the present study, HELLS expression was strictly restricted to the basal layer of the normal mucosa epithelium and that upregulation of HELLS was found in the hyperplastic basal and suprabasal layers in oral dysplasia. This pattern of HELLS expression appear highly similar to that of FOXM1 immunostaining in basal cell carcinomas³ and in HNSCC.⁷ We therefore speculate that the upregulation of HELLS (and FOXM1) in HNSCC may indicate a deregulation of progenitor cell maintenance leading to hyperproliferation and tumour development.

In summary, this study provided the first evidence that a novel set of proliferation-associated genes FOXM1, CEP55 and HELLS share progressive expression pattern during the progression of HNSCC. The fact that upregulation of these three genes precedes HNSCC malignancy renders them potential cancer biomarkers for early detection of malignant conversion. This pilot study would pave way for future studies involving more tissue samples for validating their diagnostic potential as biomarkers for HNSCC.

ACKNOWLEDGEMENTS

This study was supported by the Institute of Dentistry, Barts and The London School of Medicine and Dentistry. We thank Professor E. Ken Parkinson (Centre for Clinical & Diagnostic Oral Science) for providing SCC9, SCC15 and SCC25 cell lines and critical reading of the manuscript. We also thank Professor Ian Mackenzie (Centre for Cutaneous Research, Blizard Institute of Cell and Molecular Science) for providing POE9n, DOK, D19, D20, UK1, VB6, CaLH2, CaDec12 and 5PT cell lines.

Conflict of Interest

None declared.

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Figure Legends

Figure 1. Immunostaining of CEP55 in human normal oral mucosa, dysplasia, HNSCC and lymph node metastasis (LnMet). (A) Two representative images for each tissue category are shown here. (B) Magnified views of selected regions (dotted boxes) in (A) are shown. (C) Scatter plot of digital pixel densitometry data for CEP55 protein immunoreactivity in a panel of 20 oral tissues (n=5 in each group) as shown in (A). (D) Box-and-Whisker plot for the same data from (C). (E) Mean analysis of data points from (C) *(P<0.05) and **(P<0.01) indicate statistically significant elevation of CEP55 protein levels when compared to normal oral mucosa tissues.

Figure 2. Immunostaining of HELLS in human normal oral mucosa, dysplasia, HNSCC and lymph node metastasis (LnMet). (A) Two representative images for each tissue category are shown here. (B) Magnified views of selected regions (dotted boxes) in (A) are shown. (C) Scatter plot of digital pixel densitometry data for HELLS protein immunoreactivity in a panel of 20 oral tissues (n=5 in each group) as shown in (A). (D) Box-and-Whisker plot for the same data from (C). (E) Mean analysis of data points from (C) *(P<0.05) and ***(P<0.001) indicate statistically significant elevation of HELLS protein levels when compared to normal oral mucosa tissues.

Figure 3. Gene expression analysis of *FOXM1*, *CEP55* and *HELLS* in cell line panel and HNSCC tissues. (A) A non-linear regression analysis between *CEP55* and *FOXM1B* mRNA expression levels determined by absolute qPCR across a cell line panel consisting of 12 normal human oral keratinocytes (NHOK), 5 dysplasia and 10 HNSCC cell lines. (B) A non-linear regression analysis between *HELLS* and *FOXM1B* mRNA expression levels across the same panel of cell lines used in (A). (C) Bioinformatics analysis of *FOXM1*, *CEP55* and *HELLS* gene expression from a microarray dataset involving 4 normal oral mucosa and 16 TNM-stage defined HNSCC tissues (GDS1584²⁹). (D) Mean analysis of *FOXM1*, *CEP55* and *HELLS* gene expression using the same dataset from (C) comparing the normal (n=4) and HNSCC (n=16) samples. *(P<0.05) and **(P<0.01) indicate statistically significant gene upregulation in HNSCC when compared to normal oral mucosa tissues.

Figure 1

Figure 2

Figure 3