Title Page

SMAD4 and KCNQ3 Alterations are Associated with Lymph Node Metastases in Oesophageal Adenocarcinoma

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Highlights

- Alterations to SMAD4 and KCNQ3 are associated with altered risk of metastasis through analysis of radiologically and pathologically detected lymph node metastases.
- Both gene alterations are associated with canonical Wnt signalling, and uniquely KNQ3 alterations are associated with non-canonical Wnt signalling and altered planar cell polarity.
- Overexpression of KCNQ3 reduces wound closure in cell line assays and the number of metastases observed in xenograph models.

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Abstract

Metastasis in oesophageal adenocarcinoma (OAC) is an important predictor of survival. Radiological staging is used to stage metastases in patients, and guide treatment selection, but is limited by the accuracy of the approach. Improvements in staging will lead to improved clinical decision making and patient outcomes. Sequencing studies on primary tumours and pre-cancerous tissue have revealed the mutational landscape of OAC, and increasingly cheap and widespread sequencing approaches offer the potential to improve staging assessment. In this work we present an analysis of lymph node metastases found by radiological and pathological sampling, identifying new roles of the genes SMAD4 and KCNQ3 in metastasis. Through transcriptomic analysis we find that both genes are associated with canonical Wnt pathway activity, but KCNQ3 is uniquely associated with changes in planar cell polaritiy associated with non-canonical Wnt signalling. We go on to validate our observations in KCNQ3 in cell line and xenograph systems, showing that overexpression of KCNQ3 reduces wound closure and the number of metastases observed. Our results suggest both genes as novel biomarkers of metastatic risk and offer new potential routes to drug targeting.

Keywords: Oesophageal Adenocarcinoma, Metastasis, Mutation, Radiology, Imaging, Wnt Signalling

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Introduction

Survival of patients with oesophageal cancer remains poor and the incidence of the most common histological cell type in Europe and North America, oesophageal adenocarcinoma (OAC), has been increasing for the past forty years.[1,2] A major prognostic factor in OAC is lymph node metastases (LNMs), present in 60% of patients at diagnosis.[3,4] The presence of lymph node metastases is a significant predictor of survival in oesophageal adenocarcinoma, with overall 5-year survival reducing dramatically from 70-92% without lymph node metastases to 18-47% in patient with lymph node metastases[4].

Multi-modality radiological staging, using contrast tomography (CT), positron emission tomography (PET) and endoscopic ultrasound (EUS), is used to stage baseline nodal metastases which subsequently informs treatment decisions and prognosis.[5] Accurate assessment of lymph nodes is pivotal to complex treatment decisions, yet observational studies demonstrate the accuracy of radiological staging is poor. Subsequent management decisions are likely to result in suboptimal treatment selection for patients, which ultimately affects clinical outcomes. A majority of patients progress during treatment or develop recurrence, eventually succumbing to their disease.[6] Therefore, there is an urgent need to improve lymph node staging in OAC to optimise treatment decisions and ultimately improve patient outcomes.

Recently, the genomic landscape of primary OACs has been described in detail with whole genome sequencing (WGS) data from over 500 cases.[7] Potential driver genes have been discovered that are implicated in biological pathways associated with cancer development and prognosis. Furthermore, there is preliminary genomic evidence that genomic alteration events drive multiple sub-clones of cells from the primary OAC to form LNMs.[8] This important finding suggests that alteration driver events may initiate the development of LNMs, which if used in combination, could improve the accuracy of baseline lymph node staging, providing a more personalised approach to staging, risk stratification, and inform better treatment decisions.

Therefore, we hypothesised that alteration driver events previously described in the primary OAC lesion may also be associated with an increased risk of LNMs. In this study, we aimed to discover key driver events that significantly alter the risk of LNMs in patients with OAC using a subset of the Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) dataset used for WGS[7] that had extensive lymph node characterisation and combine this with experiments to evaluate the functional mechanisms underlying these observations.

Materials and Methods

Study Design

This prospective translational study tested multi-centre patient data before pre-clinical experiments were conducted to explore the underlying mechanisms for the observations.

Ethics

Institutional Review Board approval was granted for this study (reference 20/HCRW/0015; Sponsor Velindre University NHS Trust). Ethical approval was granted for recruitment to OCCAMS (REC 10-H0305-1). All animal studies were performed under the Animals (Scientific Procedures) Act 1986 in accordance with UK Home Office licenses (Project License 70-8823, P47AE7E47), approved by the Cancer Research UK (CRUK) Cambridge Institute Animal Welfare and Ethical Review Board.

Patient cohort

Patients with biopsy-proven oesophageal or gastro-oesophageal junction adenocarcinoma recruited to the United Kingdom (UK) OCCAMS consortium study between 2007 and 2019 were included. Patients were excluded if they were missing genetic data, staging data, or outcome data. This resulted in a cohort of three hundred and sixty eight patients for which all data was present. Fully informed written patient consent was obtained (REC 10-H0305-1).

Radiological Staging

Radiological staging followed international guidelines[5,9] and was tailored to the institution's local protocol. Patients underwent contrast-enhanced CT, followed by PET-CT +/- EUS for local staging. The Union for International Cancer Classification (UICC) Tumour Node Metastasis (TNM) 6th [10] and 7th [11] edition staging classifications were recorded for each patient because the 7th edition was adopted during the study period. Radiologists were blinded to the mutational genetic driver analysis.

Clinical Data

Clinical variables recorded included age, gender, grade of adenocarcinoma differentiation, radiological and pathological staging, oncological and surgical management, and outcomes. Gender data were submitted by the local research team. Overall survival (OS) was defined as the time from diagnosis to death from any cause, or date of last follow-up, in days. Recurrence-free survival (RFS) was defined as the time from surgical resection to recurrence or death, or date of last follow-up, in days.

Treatment

All patients underwent curative surgical resection with radical lymph node dissection. Oncological neo-adjuvant therapy was given to patients according to standard UK clinical guidelines[5] and depending on cTNM stage, perceived medical fitness and patient preference. In general, patients with at least T3 and/or N1 disease were offered neo-adjuvant platinum-based chemotherapy, with or without radiotherapy, prior to resection. Surgery was performed in specialist upper gastrointestinal cancer units.

Pathological Staging

Pathological resection specimens were reported according to the minimum recommended dataset.[12] Pathological nodal stage (pN-stage) was assigned using the TNM classification. Pathological response was defined by tumour regression grade (TRG) using the Mandard classification[13], with TRG1 indicating complete response, and TRG5 indicating no response. Pathologists were blinded to the mutational genetic driver analysis.

Genomic analysis

Procedures for obtaining the samples for genomic analysis have been described previously.[14] In summary, tissue samples were collected during diagnostic endoscopy, staging EUS examination, or intra-operatively at the time of resection. Whole genome sequencing was performed using 50x coverage with a paired germline sample. Samples were run with 150-bp paired end reads on an Illumina Hiseq4000. Events considered are copy-number alterations (CNA), single-nucleotide variants (SNV), or small insertion or deletions (indel). CNAs are described as amplified if >= 2x average ploidy of the tumour, and a loss in the event 0 copies remain. Seventy-six mutational driver genes have so far been discovered in OAC[7], we focussed on mutational driver genes with a prevalence of ≥20%. All included samples are taken from pre-treatment biopsies.

RNAseq analysis

RNAseq analysis of these patients has been described previously.[7] RNA libraries were prepared according to the illumina protocol from 250ng total RNA and sequenced using paired-end 75-bp sequencing with an Illumina HiSeq4000. For mouse RNAseq we chose to perform RNAseq on 3 control and 3 KCNQ3 OE animals (total n =6), total RNA was extracted from primary tumours using Maxwell RSC miRNA Tissue Kit (AS1460, Promega). The Illumina TruSeq stranded mRNA kit (20020595, Illumina) was used for library preparation, RNA quality confirmed using Tapestation (Agilent), quantified using Kapa qPCR library quantification kit (KK4873, Kapa Biosystems). Samples were normalised with Agilent Bravo, pooled, and sequenced on Illumina HiSeq 4000, generating paired end 100bp reads. Reads were aligned to GRCh38 with HISAT2. Reads were counted on annotated features with sub-reads featureCounts. Log2 transformed counts were generated from using the log2 function in R and counts function from DEseq2. Data from this sequencing is available at the EGA

under the following ID's: EGAN00004328220, EGAN00004328221, EGAN00004328222, EGAN00004328223, EGAN00004328224, EGAN00004328225.

Wound Closure Assays

For *in vitro* assays the oesophageal cancer cell line OE33 (RRID:CVCL_0471) was used.[15] Wound closure was assessed using a wound-healing assay using the IncuCyte system. Cells were seeded at 30,000 cells per well of a 96 well plate and grown to confluence before scratch wounds were made in each well with an IncuCyte wound maker (Essen BioScience). Twelve wells were assessed for each condition over 100 hours.

Implant models

All animal studies within the UK were performed under the Animals (Scientific Procedures) Act 1986 in accordance with UK Home Office licenses (Project License 70-8823, P47AE7E47), approved by the Cancer Research UK (CRUK) Cambridge Institute Animal Welfare and Ethical Review Board. Mouse models were generated using CD-1® (Charles River, 086) immunocompromised nude mice (RRID:MGI:5649524). No protocol was registered before the study. Implants involved OE33 WT (ctrl) or OE33 KCNQ3 OE cells. 2 x 10^5 cells were orthotopically implanted into the flanks of mice. We use 6 control, and 6 KCNQ3 overexpressing models, each mouse was considered an experimental unit. We had no exclusion criterion for removing animals during the experiments of analysis, we did not use randomisation to allocate experimental units. Mice were monitored daily for welfare changes and palpated to detect tumour growth. Mice were maintained in specific pathogen-free facility cages with access to standard diet and water and monitored for signs of tumour

formation, neurological alterations, and general welfare. Experiments were performed for up to 90 days. Full-body necropsy[16] was performed at humane end points or the maximum time point, whichever came first. All major tissues were carefully inspected for macroscopic tumour formation with the aid of direct Green Fluorescence detection.

Immunhistochemistry

Immunohistochemistry was performed using standard procedures and primary antibodies: Ki67 (RRID:AB_1547959, catalog number IHC-00375, Bethyl Laboratories, 1:1,000), cleaved caspase 3 (RRID:AB_2070042, catalog number 9664, Cell Signaling Technology, 1:200), CK5 (RRID:AB_869890, catalog number ab52635, Abcam, 1:100), CD31 (RRID:AB_2722705, catalog number 77699, Cell Signaling Technology, 1:100), α-smooth muscle actin (RRID:AB_2223021, catalog number ab5694, Abcam 1:500). Secondary antibodies were antirabbit poly-horseradish peroxidase-IgG (included in kit) or rabbit antirat (RRID:AB_10681533 , catalog number A110-322A, Bethyl Laboratories, 1:250). Digital images of entire tissue sections were captured using the Leica Aperio AT2 digital scanner (×40, resolution 0.25 µM per pixel), viewed using the Leica Aperio Image Scope v.12.3.2.8013 and quantified by HALO (Indica Labs) image analysis

Transcriptomics Analysis

Differential expression analysis was performed using the deseq2 library[17]. Genes were classed as significantly differentially expressed with adjusted p-value ≤0·05. Pathway analysis was performed using enrichr.[18] Gene set enrichment analysis (GSEA) was performed using GSEA version 4·2·1[19]. GSEA was run using hallmarks or GO: Biological Processes pathway sets and default settings using 5000 permutations, permuting the phenotype.

Patient and Public Involvement

Patient advocacy groups have been involved extensively in the OCCAMS study including commenting on clinically relevant research questions, designing patient facing materials and helping with dissemination of results to the patient community.

Statistical Analysis

Statistical analysis was performed using R version 3·6·1.[20] Continuous variables were summarised with medians and interquartile range (IQR). Categorical variables were summarised using frequencies and percentages. Relative risk of radiologically detected and pathological LNMs was calculated for each driver gene. The Benjamini-Hochberg method was used to multiple test correct p-values. A false-discovery rate was set high at 0·2 to ensure all potential genetic associations with LNMs and survival were discovered and could be further investigated. Univariable and multivariable Cox proportional hazard models tested differences in RFS and OS between gene status groups.

Cell Line Validation

Cell lines were validated using STR profiling and confirmed mycoplasma free (**appendix 13**)

Role of Funders

Funders played no role in the study design, data collection, data analysis, data interpretation, or the writing of this manuscript.

Results

Patient cohort characteristics to investigate drivers of lymph node metastases in oesophageal adenocarcinoma

Three hundred and sixty-eight patients with radiological and pathological LNM data, coupled with genomic and transcriptomic analysis, were included. We first tested alterations in the most recurrently mutated driver genes previously described in OAC[7] for association with radiological (combined CT, PET and EUS) and pathological (resection specimen) metastases, because these assessments occur at different times in the treatment pathway. We then performed transcriptomic analysis coupled with study of *in vitro* and *in vivo* metastasis models to validate and assess molecular mechanisms of LNMs in OAC. (**Figure 1**)

Patients were recruited from twenty sites. Median patient age was 67·0 years (inter-quartile range (IQR) 59·3-73·5). Median RFS and OS were 1037 days (IQR 452-2975) and 1238 days (IQR 580-2421), respectively. Patients were followed-up until 5 years after diagnosis, or death. Baseline characteristics of included patients are detailed in **appendix 1**. CONSORT flow diagram is shown in **appendix 2**. Diagnostic test accuracy of individual CT, PET-CT and EUS staging investigations, using pathological staging as reference standard, is included in **appendix 3**.

To test associations between metastases and genomic events in patients, we first defined the most frequent driver events in the cohort. We pre-specified the criterion to study alterations (copy number alterations or nonsynonymous mutations) in driver genes discovered by

Frankell et al[7] with a penetrance of 20% or more. Ten genes met this criterion; TP53 (76·4%), CDKN2A (44·0%), KDM6A (43·8%), SMAD4 (37·0%), CCDC102B (28·5%), KCNQ3 (26·4%), PCDH17 (23·4%), GATA4 (21·5%), KRAS (21·5%), CHL1 (20·4%) (**Figure 2**). Five genes (TP53, CDKN2A, KRAS, GATA4, SMAD4) were also identified in the ten most prevalent mutations in Frankell et al[7]. Four have previously been described in pathways to metastasis (CDKN2A, GATA4, KRAS, and TP53).[8] The frequency of the seventy six mutational driver genes previously described in Frankell et al[7] are listed in **appendix 4**.

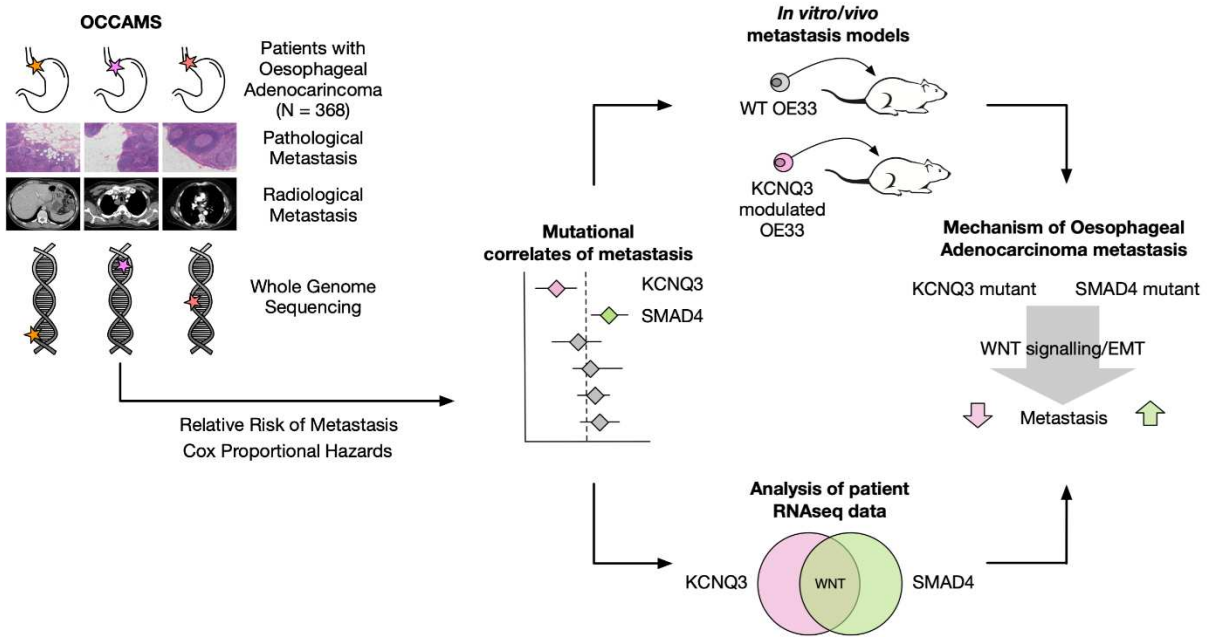


Figure 1: Identification of molecular correlates and mechanisms of lymph node metastases in oesophageal adenocarcinoma. Patient data from the OCCAMS dataset with available radiological and pathological data (n=368) were compared with whole genome sequencing to test molecular associations of lymph node metastases. Having identified molecular associations, RNAseq data was analysed alongside *in vitro* motility assays and *in vivo* metastasis models using manipulated OE33 cell lines to validate findings and identify molecular mechanisms. EMT, epithelial to mesenchymal transition.

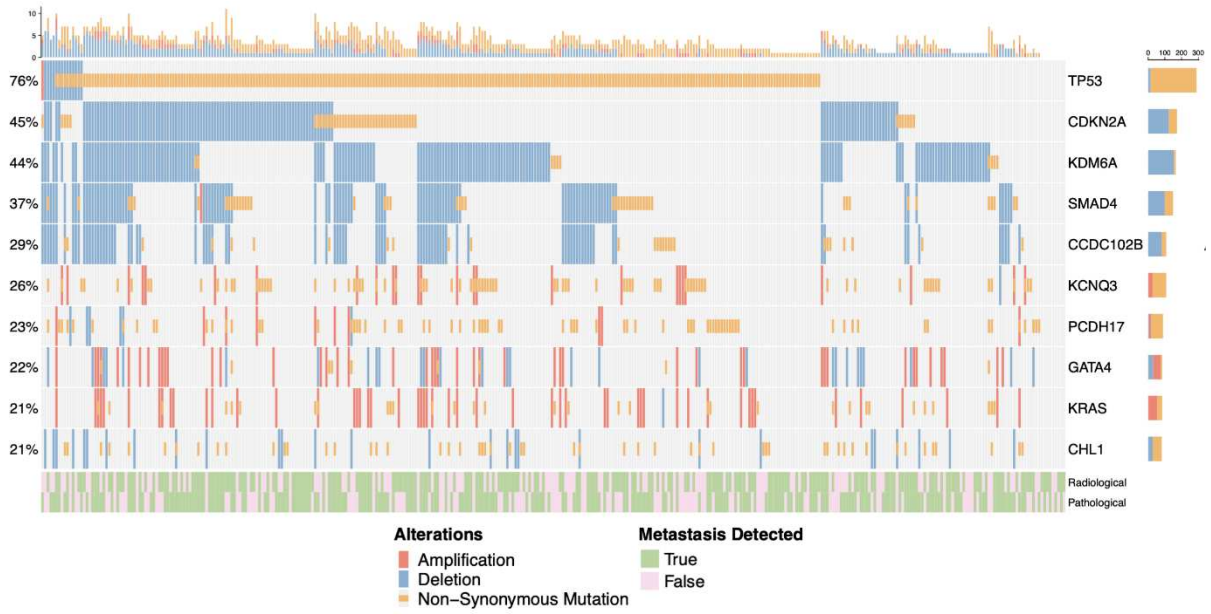
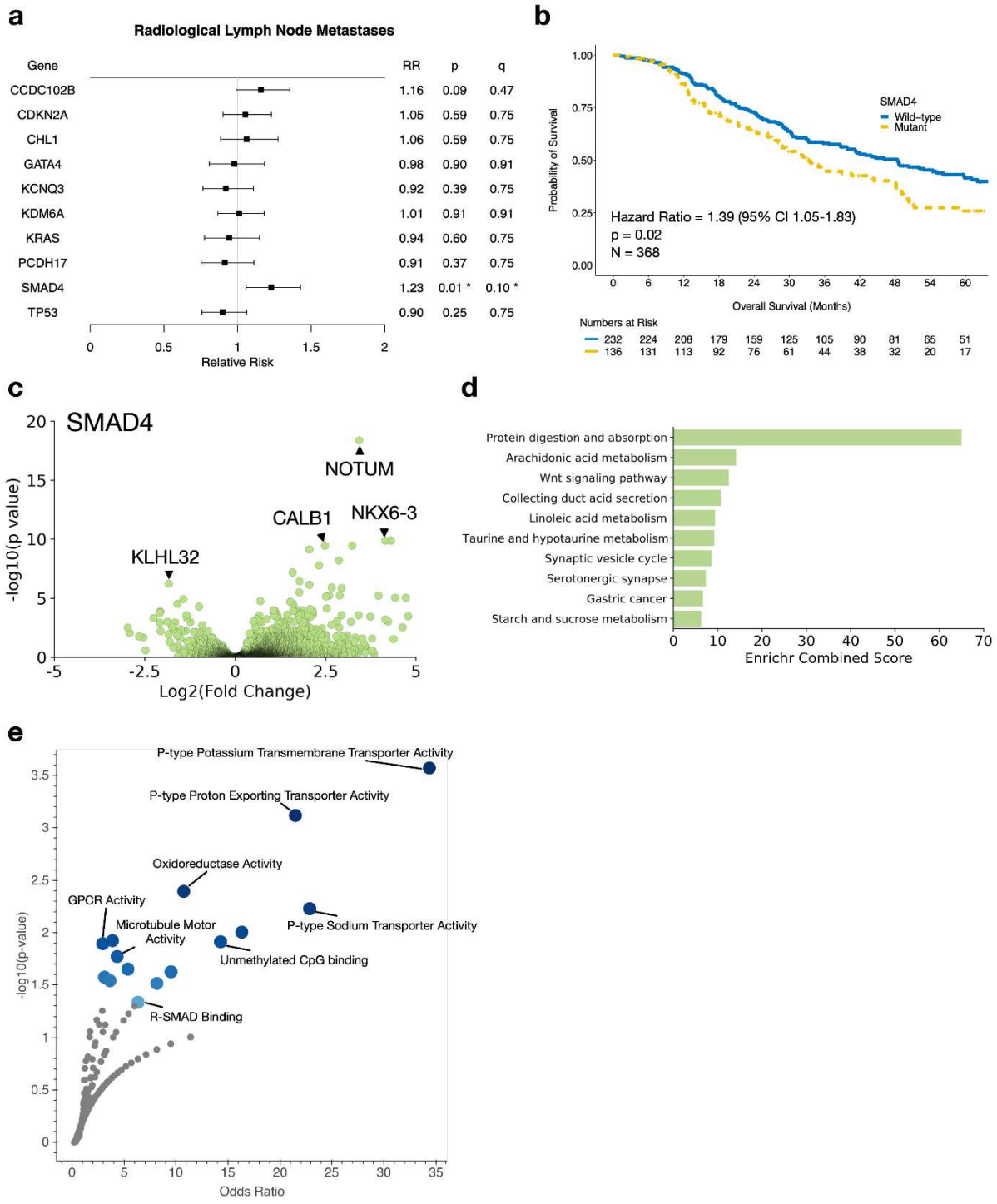


Figure 2. Ten most frequent driver events in patient cohort with presence of radiologically detected and pathological lymph node metastases for each of the 368 oesophageal adenocarcinoma patients. SNVs, Indels or CNVs are shown for each patient. Amplification was defined as copy-number-adjusted ploidy >= 2× the average ploidy of that tumour and a loss in the event 0 copies of a gene remained. On the right, the percentages of different SNV/Indels and CNAs are shown. Above the plot, the number of driver mutations per sample is shown.

Alteration to SMAD4 predicts radiological metastasis status in oesophageal adenocarcinoma

We first tested the relative risk of radiological LNMs against all prevalent mutational driver genes. SMAD4 alteration was the only event significantly associated with increased risk of radiologically detected LNMs (relative risk (RR) 1·23, 95% CI 1·06-1·43, log-rank p=0·01), and remained significant after adjustment for multiple comparisons (**Figure 3a**). SMAD4 alteration was also significantly associated with patient survival - a shorter RFS (HR 1·33, 95% CI 1·01-1·75, p=0·05) and shorter OS (HR 1·39, 95% CI 1·05-1·83, log-rank p=0·02) (**Figure 3b**), in line with previous work[7,21]. No significant difference in relative risk for radiological LNMs was found for the other nine mutational driver genes tested (**appendix 5**).

To explore the molecular mechanisms related to alteration of SMAD4 in OAC, we analysed available transcriptomic patient data. We performed differential expression analysis, then pathway enrichment analysis on differentially expressed genes between patients WT (n = 144) and altered (n = 79) for SMAD4. Three hundred and sixty-three genes were differently expressed between SMAD4 altered and WT patients (wald $q \leq 0{\cdot}05$). The most significantly upregulated gene is the secreted protein NOTUM (**Figure 3c**), known regulator of Wnt and previously shown to be upregulated in adenocarcinomas, correlating with tumour initiation and progression.[22,23] Molecular analysis of KEGG pathways applied to these genes identified significant enrichment for protein digestion and absorption, Wnt signalling, and metabolic pathways, consistent with a metastatic effect (**Figure 3d**). Gene Ontology (GO) molecular function pathways identified SMAD signalling and microtubule motor signalling. It also identified several gene sets involved in ionic transport of sodium and potassium across membranes (**Figure 3e**), potentially indicating a role for ion channels and membrane potential in OAC metastases.



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329 **Figure 3: SMAD4 alterations correlate with radiological metastasis risk in OAC and induce**
330 **changes in the Wnt pathway.** a) Forest plot of top 10 significant correlates of radiological
331 lymph node metastasis from 72 identified driver genes. RR relative risk. *log-rank p<0·05. *
332 log-rank q<0·2 after Benjamini-Hochberg adjustment. b) Kaplan-Meier analysis for overall
333 survival of SMAD4 mutant and WT patients. N = 368 c) Volcano plot of the differentially

expressed genes between SMAD4 altered and WT patients. d) Pathway enrichment for KEGG pathways in SMAD4 mutant patients. e) Pathway enrichment for gene ontology (GO): Molecular Function pathways in differentially expressed genes from SMAD4 mutant vs WT patients.

Genomic alteration of potassium ion channel KCNQ3 predicts pathological metastases in oesophageal adenocarcinoma

We next tested the relative risk of *post-treatment pathological LNMs* against the same list of ten prevalent mutational driver genes. KCNQ3 alteration was the only event significantly associated with a risk of pathological LNMs, predicting a reduced risk (RR 0·78, 95% CI 0·64-0·96, log-rank p=0·01) and remaining significant after adjustment for multiple comparisons (**Figure 4a**). No significant difference in relative risk for pathological LNMs was found for the other nine mutational driver genes, including SMAD4 (**appendix 6**). No significant different in OS was found between KCNQ3 alteration and WT patients (HR 0·98, 95% CI 0·72-1·33, log-rank p=0·90).

However, in non-responders, who were overall staged as cN0 after a combination of radiological investigations (n=61), there was separation of survival curves between KCNQ3 mutant and WT groups, but this did not reach statistical significance (HR 0.47 (95% CI 0.19-1.13), log-rank p=0.09) (**appendix 7**). Further, we tested the relative risk of lymph node metastases in the largest sub-group of patients who were treated with neo-adjuvant epirubicin, cisplatin, capecitabine (ECX) therapy. In the 146 patients treated with ECX, similar

results were obtained compared to the overall patient cohort. The relative risk of lymph node metastases with KCNQ3 alteration was 0.74 (95% CI 0.51-1.08), log-rank p=0.077.

Given we previously identified SMAD4 as a significant predictor of radiologically detected LNMs, we tested the added predictive value of SMAD4 to KCNQ3 alterations for pathological LNMs and built a multi-variable logistic regression (**appendix 8**). Clinical N-stage (odds ratio (OR) 2·29 95% CI 1·24-4·27, log-rank p=0·009) and KNCQ3 (OR 0·46 95% CI 0·24-0·89,log-rank p=0·022) remained independently associated with LNMs compared to currently used clinical factors.

To explore altered cellular pathways in our patients, we performed transcriptomics analysis of KCNQ3 altered (n = 62) and WT (n = 161) patients. Differential expression identified two hundred and sixty one significantly altered genes (**Figure 4b**), including PTH2R and NKD1, genes known to interact with Wnt signalling. KEGG enrichment identified Wnt signalling as the most significantly enriched pathway in this set of genes (**Figure 4c**), several metabolic disruptions and, overlapping with SMAD4 alterations, protein digestion and absorption.

As our analysis included all nonsynonymous mutations and copy number changes rather than just missense mutations, it resulted in a larger cohort of patients being described as altered for KCNQ3 than previous work (26·4% vs 9·1% in Frankel et al). Patients mutant for KCNQ3 generally have nonsynonymous mutations (81/97 - 83·5%, **Figure 4d**), the majority of which are 3'UTR (49/81 – 60·5%, **Figure 4e**), missense (19/81 – 23·5%), or both (8/81 - 9·9%). Reanalysis of pathological metastasis associations with these subsets of mutations (3'UTR or Missense, **Figure 4f**) confirms that both mutation types are independently significantly

associated with a reduction in pathological metastasis risk (3'UTR; RR 0·71 95% CI 0·53-0·97 log-rank p=0·01. Missense; RR 0·61 95% CI 0·345-1·087 log-rank p=0·03). Patient RNAseq also confirms similar differentially expressed genes (**Figure 4g**), and overlapping GO Biological Pathways (**Figure 4h**), demonstrating that 3'UTR mutations of KCNQ3 are biologically similar to missense mutations. These pathways include those involved in epidermal development, keratinization, and epithelial cell differentiation, consistent with an effect on metastatic potential.

To investigate the overlap between alterations to SMAD4 and KCNQ3 in OAC patients, we performed comparative analysis of patients altered for each gene compared to those WT for both. We found twenty one overlapping significantly differentially expressed genes (**appendix 9**). STRING analysis shows these genes mainly link to the Wnt transcription factor beta-catenin. GSEA analysis against the hallmarks gene set demonstrates that these patients display a remarkably similar upregulation of cancer progression-associated pathways, including Wnt signalling.

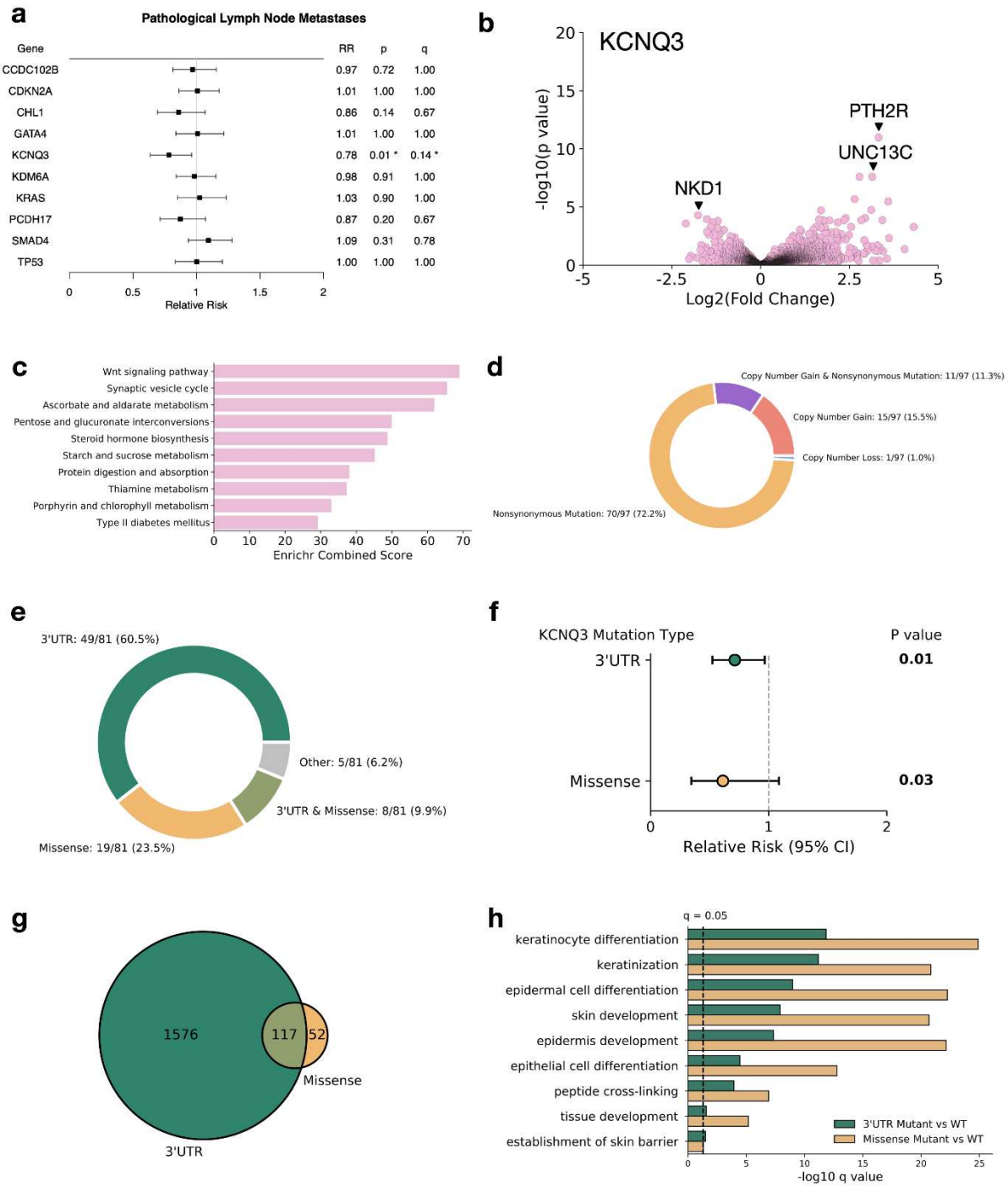


Figure 4: KCNQ3 alterations are associated with pathological lymph node metastasis risk in oesophageal adenocarcinoma and induce changes in the Wnt pathway. a) Forest plot of top 10 significant correlates of pathological lymph node metastases from 72 identified driver genes. RR relative risk. * log-rank p<0·05. * log-rank q<0·2 after Benjamini-Hochberg adjustment. b) Volcano plot of the differentially expressed genes between KCNQ3 altered

and WT patients. c) Pathway enrichment for KEGG pathways in KCNQ3 altered patients. d) KCNQ3 alteration types in our cohort. e) Nonsynonymous KCNQ3 mutation types in our cohort. f) Forest plot for 3'UTR and missense mutations in KCNQ3. g) Overlapping differentially expressed (wald $q < 0.05$) genes for patients with 3'UTR mutations in KCNQ3 vs WT KCNQ3, and patient with missense mutations in KCNQ3 vs WT KCNQ3. h) Overlapping enriched GO: Biological Pathways between patients with 3'UTR mutations in KCNQ3 and missense mutations in KCNQ3.

In vivo models suggest a role for KCNQ3 in oesophageal adenocarcinoma metastases through altering cell polarity

Whilst alterations to SMAD4 have been identified previously as driving metastasis[21], for KCNQ3 alterations there is an apparent paradox whereby mutations in patients appear to increase Wnt/MYC signalling, but reduce the probability of metastases. To clarify this, we investigated previously used models of OAC cells overexpressing KCNQ3. OE33 cells overexpressing KCNQ3 proliferate faster, increase Wnt signalling, and transcriptionally alter a large subset of pathways that are also altered in patients[24]. We explored how these cell lines are altered in their ability to metastasise *in vitro* and *in vivo*.

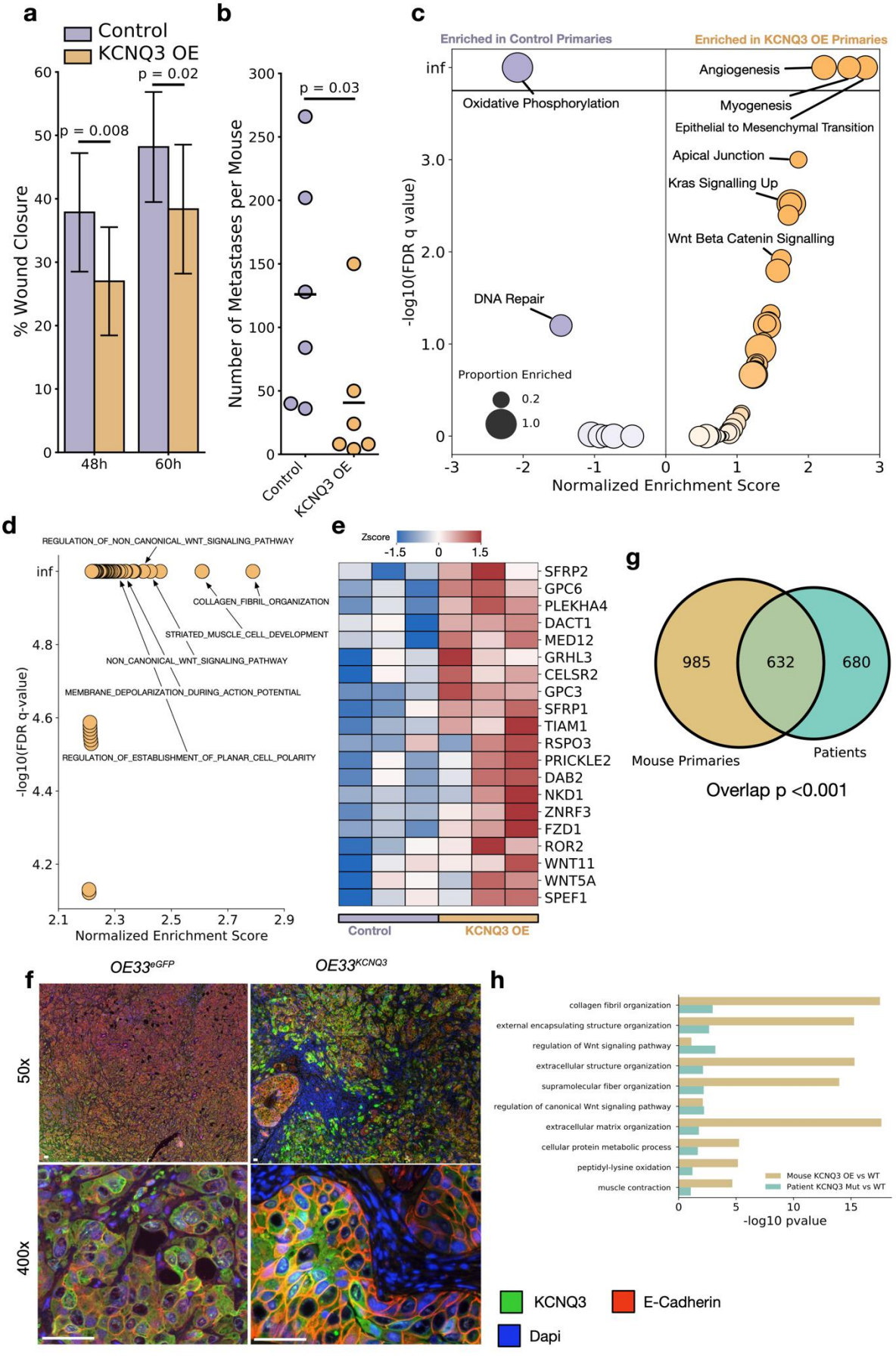
Live cell wound healing assays found that, despite increasing proliferation rate of OE33, KCNQ3 overexpressing cells exhibited reduced motility and ability to close gaps in Matrigel (Students t-test $p < 0.05$) (**Figure 5a**). We next implanted OE33 cells wildtype (WT) and overexpressing (OE) KCNQ3 orthotopically into the flanks of nude mice to study metastasis. After reaching endpoint size, necropsy was performed to look for metastases. Consistent with

patient and *in vitro* models, we found a significantly reduced number of metastases (Wilcoxon p<0·05) in models with KCNQ3 overexpressing cells compared to WT (mean number of metastases: WT – 126.0±84.3, KCNQ3 OE – 40.7±50.3) (**Figure 5b**). Despite this, primary tumor growth over the first 50 days was increased in KCNQ3 OE implants (**appendix 10**) – consistent with previous findings.

RNAseq analysis of these primary tumors (**Figure 5c**) identifies upregulation of angiogenesis, myogenesis, and epithelial to mesenchymal transition (EMT) pathways; traditionally associated with increased metastasis. We also observed increased Beta-catenin signalling, consistent with previous work[24]. We previously identified increased planar cell polarity signalling in OE33 cells overexpressing KCNQ3[24], and so hypothesised that the increase in cell polarity signalling may impact cellular ability to metastasise. GSEA against the GO: Biological Processes gene sets confirms a significant enrichment for gene sets involved in cell polarity and non-canonical Wnt signalling (**Figure 5d, e**).

We also observed that KCNQ3 OE cell lines upregulated cadherins, including P, E, and N-cadherin, associated with EMT. Immunofluorescence confirms the presence of E-cadherin and an epithelial phenotype in KCNQ3 OE OE33 primary tumours (**Figure 5f**), (single channels shown in **appendix 11**) and previous work has highlighted the pivotal role E-cadherin plays in OAC metastasis[25]. Immunohistochemistry also reveals an increase in protein levels of cytokeratins (CK5/6) and metastasis suppressor coiled-coiled protein 3 (CC3), and a decrease in cell adhesion molecule PECAM-1 (CD31), consistent with reduced metastasis (**appendix 12**). Despite KCNQ3 OE increasing Wnt activity and triggering a transcriptional change consistent with metastasis, this does not correlate with increased metastasis *in vitro* or *in vivo*.

Finally, we compared gene expression profiles from KCNQ3 altered vs WT OAC patients, with KCNQ3 OE versus WT OE33 implant model. There was significant overlap (Fishers one-tailed p<0·05) between enriched GO terms, with 48% of pathways from patients and 39% from OE33 models overlapping (**Figure 5g**). The top 10 (ranked by mean p-value) overlapping pathways between the two models included Wnt signalling and collagen/extracellular matrix organisation, suggesting that OE33 KCNQ3 OE versus WT implant models change similar signalling pathways to KCNQ3 altered versus WT patients (**Figure 5h**).


a
Control
KCNQ3 OE
% Wound Closure
p = 0.008
p = 0.02
48h
60h
b
Number of Metastases per Mouse
p = 0.03
Control
KCNQ3 OE
c
Enriched in Control Primaries
Enriched in KCNQ3 OE Primaries
-log10(FDR q value)
Oxidative Phosphorylation
Angiogenesis
Myogenesis
Epithelial to Mesenchymal Transition
Apical Junction
Kras Signalling Up
Wnt Beta Catenin Signalling
DNA Repair
Proportion Enriched
0.2
1.0
Normalized Enrichment Score
d
REGULATION_OF_NON_CANONICAL_WNT_SIGNALING_PATHWAY
COLLAGEN_FIBRIL_ORGANIZATION
STRIATED_MUSCLE_CELL_DEVELOPMENT
NON_CANONICAL_WNT_SIGNALING_PATHWAY
MEMBRANE_DEPOLARIZATION_DURING_ACTION_POTENTIAL
REGULATION_OF_ESTABLISHMENT_OF_PLANAR_CELL_POLARITY
-log10(FDR q-value)
Normalized Enrichment Score
e
Zscore
SFRP2
GPC6
PLEKHA4
DACT1
MED12
GRHL3
CELSR2
GPC3
SFRP1
TIAM1
RSPO3
PRICKLE2
DAB2
NKD1
ZNRF3
FZD1
ROR2
WNT11
WNT5A
SPEF1
Control
KCNQ3 OE
g
985
632
680
Mouse Primaries
Patients
Overlap p <0.001
f
OE33eGFP
OE33KCNQ3
50x
400x
h
collagen fibril organization
external encapsulating structure organization
regulation of Wnt signaling pathway
extracellular structure organization
supramolecular fiber organization
regulation of canonical Wnt signaling pathway
extracellular matrix organization
cellular protein metabolic process
peptidyl-lysine oxidation
muscle contraction
Mouse KCNQ3 OE vs WT
Patient KCNQ3 Mut vs WT
-log10 pvalue
KCNQ3
E-Cadherin
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460

Figure 5: KCNQ3 expression negatively impacts OAC metastasis *in vitro* and *in vivo*. a) Wound closure assay at two timepoints for OE33 WT and KCNQ3 OE OE33 cell lines. P-values represent students t-test. * p<0·05, ** p<0·01. N = 5 repeats. b) Number of metastases per mouse for KCNQ3 OE OE33 implant models vs OE33 WT. p-values represent Wilcoxon test. * p<0·05. N = 6 repeats. c) GSEA pathway analysis on RNAseq from the primaries of KCNQ3 OE OE33 and OE33 WT. d) Top 50 pathways enriched in KCNQ3 OE OE33 primaries against the GO: Biological Processes gene sets. e) Heatmap of genes enriched in GO: Regulation of Establishment of Planar Cell Polarity for KCNQ3 OE OE33 and OE33 WT primaries. f) Staining for expression of E-Cadherin in orthotopic OE33 primaries. Scale bar represents 50um. g) Overlapping GO biological processes between KCNQ3 OE OE33 vs WT mouse primaries, and KCNQ3 altered vs WT patients. h) Top 10 significant pathways overlapping between KCNQ3 OE OE33 vs WT mouse primaries and KCNQ3 altered vs WT patients.

Discussion

We have demonstrated that genomic alterations in SMAD4 and KCNQ3 are associated with LNMs in OAC with concordant findings in patient-derived multi-omic data, *in vitro* cell culture, and *in vivo* metastasis models. SMAD4 alteration was the only genomic event associated with an increased risk of radiologically detected LNMs, whereas KCNQ3 alteration was the only event associated with a reduced risk of pathological metastasis. Furthermore, significant differences in survival (RFS and OS) were demonstrated between SMAD4 altered and WT patients. Both alterations increase canonical Wnt signalling, but the apparent paradox that SMAD4 alterations associate with increased metastases, whilst KCNQ3 alterations reduce

metastases, can be explained by an observed increase in non-canonical Wnt (planar cell polarity) signalling by KCNQ3 alterations.

Whilst SMAD4 has previously demonstrated significant roles in OAC disease progression and survival[7,21,26], as well as being implicated in metastases in other gastrointestinal cancers[27,28], here we explicitly link SMAD4 alteration to radiologically detected LNMs in OAC. Furthermore, KCNQ3 is a newly identified genomic driver in OAC[7,24], this prompted us to study KCNQ3 activity both in *vitro* and *vivo* in order to establish the validity of this finding. Whilst KCNQ3 alterations appear to be under selection and increase the proliferative ability of the primary tumour, this work suggests that this progression does not correlate with increased metastatic propensity, but actually reduces the likelihood of a metastatic event, possibly through up-regulation of planar cell polarity pathways. This adds to the emerging work in other tissues where KCNQ genes have been identified as impacting phenotype and patient outcome[1,2], and the increasing importance ion channels play in cancer[31]. This also highlights the variability and tissue specificity of ion channel activity, whereby findings based on a different member of the same family in a different tissue, KCNQ1 in colorectal adenocarcinoma, implicate it as a tumor suppressor. This work also highlights the importance of studying different stages of the tumor lifespan, as our implant models show KCNQ3 overexpressing cells grow faster than their WT equivalents, and so would be expectd to outcompete them in a heterogeneous tumor – however, our finding highlight that whilst these tumors may grow faster, they would be expected to metastasise less. Our findings also support the previously reported[32] and increasing importance of E-cadherin in OAC metastases since E-cadherin expression remained high in the primary tumours, and correlated with a reduction in metastases.

Accurate staging of lymph node metastases in oesophageal adenocarcinoma is vitally important to complex treatment decisions in many patients. In cases where primary tumours are potentially resectable, and at an early stage (T2 or less), the presence of lymph node metastases will change a patient's treatment from surgery alone to the addition of pre-operative therapy (either chemotherapy or chemoradiotherapy), depending on clinical factors. Further, the location of lymph node metastases is crucial. Neo-adjuvant chemotherapy and chemoradiotherapy are both effective regimens for pre-operative treatment and are selected depending on patient factors, and crucially, the radiological staging. However, the decision on suitability of radiotherapy is influenced by the location of any lymph node metastases and, in particular, the length of disease determined by radiological staging. If a lymph node metastasis is present outside of the maximum encompassable radiotherapy field, then radiotherapy is not possible. Similarly, if a lymph node metastasis is located outside of the curative surgical resection field, then radical oesophagectomy is not attempted.

Despite the current reliance placed on imaging, there is a pressing need to improve nodal staging because the accuracy of radiological lymph node assessment is poor. Radiological techniques are insensitive to small lymph node metastases that harbour within normal sized lymph nodes. Diagnostic test accuracy studies have shown that the sensitivity of CT, EUS and PET was 39.7%, 42.6% and 35.3%, respectively and the specificity was 77.3%, 75.0% and 90.9%, respectively[33]. Current imaging methods cannot differentiate these from normal lymph nodes.

In this cohort, SMAD4 and KCNQ3 alterations were prevalent in more than 20% of patients. However, we note that our analysis identified an increased number of alterations in both SMAD4 and KCNQ3 compared to previous work,[7] mainly because of our inclusion of all nonsynonymous mutations. We demonstrate however, the 3'UTR mutations in KCNQ3 behave similarly to missense mutations, and as such highlight that these poorly understood and studied mutational subtypes are of strong biological relevance in OAC. The clinical benefit of these markers is likely to be found in patients with normal or borderline sized lymph nodes on imaging. In this setting, confirmation of SMAD4 or KCNQ3 mutational status obtained via haematoxylin and eosin (H&E) staining could indicate that the probability of lymph node metastases is high and add evidence to support a change in treatment selection.

There are limitations of this work. A prospective study is necessary to evaluate and compare the utility of a genomic-enhanced staging pathway against standard practice of basing staging decisions based entirely on radiological and pathological parameters before or after surgical treatment respectively. Integration of genomic analysis into staging adds complexity and cost, but this could be mitigated with the option to use immunohistochemistry or other methods for detection of genomic drivers. We analysed radiological and pathological LNMs in parallel because these staging assessments occur at different timepoints and cannot be directly compared given the impact of neoadjuvant treatment. Though we found that SMAD4 alteration increases the relative risk of radiologically-detected LNMs and has prognostic significance, the diagnostic accuracy of radiological staging was suboptimal, a finding previously reported.[33] Further work should explore the value of combining radiological staging and genomic analysis. Further, we note that all transcriptomics analysis performed in

this study was done on primary tumours, and further work should attempt to sample and study metastases to confirm and expand these findings.

In conclusion, we have discovered two molecular correlates of LNMs in OAC, SMAD4 and KCNQ3. We used high-quality prospectively collected patient data and confirmed these findings using transcriptomic analysis coupled with study of *in vitro* and *in vivo* metastasis models. Patients with SMAD4 alterations have increased risk of radiologically detected LNMs which has prognostic significance. In contrast, patients with KCNQ3 alterations have a lower risk of pathological LNMs by significantly increasing non-canonical Wnt signalling. These important findings could facilitate a personalised approach to radiological staging, leading to improved risk stratification, more informed treatment decisions, and ultimately better patient survival.

Contributors

KGF and DS designed the study. KGF coordinated the study. DS performed all genomics analysis and directed experimental validation. ER performed *in vivo* and *in vitro* validation, LZ cultured OE33 cell lines and performed genetic manipulation. KGF and DS performed the statistical analyses. RCF and BAH supervised the study. KGF and DS have both independently verified the underlying data in this study. KGF and DS wrote the original draft manuscript. All authors critically revised the manuscript, had full access to data in this study, and had final responsibility for the decision to submit for publication. All authors have read and approved the final version of this manuscript.

Professor Rebecca Fitzgerald acts as guarantor for the study.

Data Sharing Statement

The results from data generated in this study are available in the appendix (tables and figures), and data is available at the EGA IDs: EGAN00004328220, EGAN00004328221, EGAN00004328222, EGAN00004328223, EGAN00004328224, EGAN00004328225.

Declaration of Interests

RCF is named on patents related to Cytosponge and related assays which have been licensed by the Medical Research Council to Covidien GI Solutions (now Medtronic) and is a co-founder of CYTED Ltd. The other authors declare no competing interests.

Acknowledgements

KGF receives research funding from the Moondance Foundation at Velindre Cancer Centre and Health and Care Research Wales (HCRW), DS is funded through a grant from the Medical Research Council held by BAH (grant no. MR/S000216/1). RJG and ER are supported by Cancer Research UK. BAH acknowledges support from the Royal Society (grant no. UF130039) and MRC (grant no. MR/S000216/1). The laboratory of RCF is funded by a Programme Grant from the Medical Research Council (MR/W014122/1, G111260), and acknowledges funding from CRUK ECMC and NIHR BRC. OCCAMS was funded through CRUK (RG81771/RG84119, A22720/A22131).

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Funding

No direct funding was received for this study. This work was not commissioned.

KGF receives research funding from the Moondance Foundation at Velindre Cancer Centre and Health and Care Research Wales (HCRW), DS is funded through a grant from the Medical Research Council awarded to BAH (grant no. MR/S000216/1). RJG and ER are supported by Cancer Research UK. BAH acknowledges support from the Royal Society (grant no. UF130039) and MRC (grant no. MR/S000216/1). The laboratory of RCF is funded by a Programme Grant from the Medical Research Council (MR/W014122/1, G111260). OCCAMS was funded by a Programme Grant from Cancer Research UK (RG66287, A15874). OCCAMS2 was funded by a Programme Grant from Cancer Research UK (RG81771/RG84119, A22720/A22131).

Patient consent for publication

Not required.

766