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Elevated Tumor Expression of PAI-1 and SNAI2 in Obese Esophageal Adenocarcinoma Patients and Impact on Prognosis

Emma H. Allott, PhD[1], Melissa J. Morine, PhD[2,3], Joanne Lysaght, PhD[1], Sarah A. McGarrigle, PhD[4], Claire L. Donohoe, MB, BCh, BAO, MRCS[1], John V. Reynolds, MD, FRCS[1,4], Helen M. Roche, PhD[2] and Graham P. Pidgeon, PhD[1,*]

OBJECTIVES: Obesity is linked to increased mortality from many cancer types, and esophageal adenocarcinoma (EAC) displays one of the strongest epidemiological associations. The aims of this study are to dissect molecular pathways linking obesity with EAC and to determine if obesity is linked to increased aggressiveness of this disease.
METHODS: Affymetrix microarrays identified altered signaling pathways in an EAC cell line following coculture with visceral adipose tissue or isolated adipocytes from viscerally obese EAC patients ($n = 6$). Differentially expressed genes were subsequently investigated in patient tumor biopsies by quantitative reverse transcriptase PCR and examined with respect to obesity status, tumor biology, and patient survival.
RESULTS: Visceral adipose tissue induced expression of genes involved in epithelial mesenchymal transition (EMT), plasminogen activator inhibitor (PAI)-1, and transcription factor SNAI2, in an EAC cell line. In EAC patient tumor biopsies from obese patients, we noted elevated expression of these genes, together with reduced expression of epithelial marker E-cadherin. SNAI2 was associated with EAC prognosis.
CONCLUSIONS: Expression of EMT genes, PAI-1 and SNAI2, was elevated in tumors of obese EAC patients, and SNAI2 was associated with poor survival. Genes deregulated in obesity and associated with prognosis may represent potential targets for treatment stratification of obese EAC patients.

INTRODUCTION

Overweight and obesity are linked to increased incidence and mortality from multiple cancer types,[1] with esophageal adenocarcinoma (EAC) displaying one of the strongest epidemiological associations.[2] The incidence of this aggressive cancer has risen by 50% in the last 15 years,[3] mirroring the alarming rise in obesity, and over 40% of cases of EAC are now estimated to be directly attributable to excess adiposity.[4] EAC is a highly lethal disease due in part to early malignant tumor cell dissemination,[5] indicating that pathways of tumor invasion and metastasis may be of particular importance. In this study, we sought to investigate if these pathways are modulated by obesity in EAC.

In addition to its role as a proteolysis inhibitor, plasminogen activator inhibitor 1 (PAI-1) has a direct role in cell attachment and detachment to vitronectin through competition with cell surface integrins and uPAR for vitronectin binding sites, thus modulating tumor cell migration.[6] Treatment of tumor cells with exogenous PAI-1 has been found to induce a dose-dependent decrease in vitronectin binding and a corresponding increase in migratory and invasive capacity, with these effects abrogated by an antibody against PAI-1.[7] PAI-1 is highly induced in SNAIL, SNAI2, and E47-transfected epithelial cells undergoing epithelial mesenchymal transition (EMT)[8] and is the most prominently induced transcript in the TGF-β1 and EGF-induced "scatter" phenotype in malignant human keratinocytes,[9] indicating an important role for this extracellular matrix remodeling protein in the process of EMT. In addition, PAI-1 is a circulating adipokine, elevated in obesity, and with a direct role in the development of insulin resistance.[10] Inhibition of PAI-1 could therefore be particularly relevant in viscerally obese patients with raised circulating levels of this adipokine.

The process of EMT is required for tumor cells to acquire a migratory phenotype via altered expression of cell adhesion molecules and reorganization of the cytoskeleton. SNAI2 is a well-established transcriptional regulator of this process acting often, but not always, via repression of the epithelial cell adhesion molecule E-cadherin.[11] In addition, expression of SNAI2 in adipose tissue is required for adipogenesis, and SNAI2-overexpressing mice display adipocyte hypertrophy, while adipose tissue mass is reduced in SNAI2-deficient mice.[12] SNAI2 signaling thus represents a common pathway in both adipose tissue and tumor cells, indicating its potential as a novel therapeutic target in obesity-fuelled cancers.

The aim of this study was to investigate molecular mechanisms underlying the association of obesity and EAC,

[1]Department of Surgery, Trinity Centre for Health Sciences, St James's Hospital, Dublin 8, Ireland; [2]Nutrigenomics Research Group, UCD Conway Institute and School of Public Health, UCD, Dublin 4, Ireland; [3]The Microsoft Research-University of Trento Centre for Computational Systems Biology (COSBI), Rovereto, Italy; [4]St James's Hospital, Dublin 8, Ireland.
*Correspondence: Graham P. Pidgeon, PhD, Department of Surgery, Trinity Centre for Health Sciences, St James's Hospital, Dublin 8, Ireland. E-mail: pidgeong@tcd.ie
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based on the hypothesis that adipose tissue would differentially affect the expression of pro-tumor pathways in EAC cells. Affymetrix microarray technology determined the effect of coculturing EAC cells with adipose tissue and adipocytes, and the expression of genes identified using this coculture system was subsequently investigated in patient tumor biopsies with respect to obesity status, tumor biology, and survival. For the first time, we report increased expression of *SNAI2* and *PAI-1* in the tumors of obese EAC patients, with *SNAI2* significantly associated with poor prognosis. The identification of gene expression pathways responsible for obesity-fuelled cancers could present novel therapeutic targets for stratified treatment of obese cancer patients.

METHODS

Patient cohort. All patients gave informed consent for use of their tissue in this study and the study obtained ethical approval from St James's Hospital Ethical Review Board (Table 1). EAC patients were separated into viscerally obese and non-obese categories by waist circumference (WC) according to the International Diabetes Federation criteria,[13] by body mass index (BMI) $\geq 30 \, \text{kg/m}^2$, or by visceral fat area (VFA). There currently exists no standard cutoff value for VFA and cancer risk. We therefore used a VFA $\geq 130 \, \text{cm}^2$, established as a cutoff for metabolic risk of CVD[14,15] and previously published by our group in EAC patients.[16]

Tissue preparation. Tumor biopsies from EAC patients were immediately taken from fresh tumor tissue following surgical resection and processed by a designated biobank technician, using standardized operating procedures in order to procure consistently high-quality biospecimens, as previously published.[17] Visceral adipose tissue specimens were excised at the commencement of surgery and immediately transported in transport buffer (phosphate-buffered saline, glucose (0.1%), and gentamycin (0.05 mg/ml)) for processing. To generate adipose tissue-conditioned medium, an adapted protocol from Fried and Moustaid-Moussa[18] was used. Briefly, visceral adipose tissue was minced, washed in phosphate-buffered saline, and cultured in M199 medium (ISIS, Wicklow, Ireland) (containing 0.05 mg/ml gentamycin) at a ratio of 5 g adipose tissue to 10 ml medium for 72 h, aliquotted, and stored at $-80 \, °C$ until analyzed. Visceral adipose tissue (10 g) was digested with collagenase buffer (2 mg/ml Type II collagenase (Sigma, Wicklow, Ireland), 4% bovine serum albumin (Sigma) in Krebs–Ringer bicarbonate buffer, ISIS, Wicklow, Ireland, pH 7.4), and extracted adipocytes were ceiling cultured at a dilution of 250,000 cells per well as previously described.[19]

Cell culture. The OE33 cell line, derived from EAC (ECACC, Health Protection Agency Culture Collections, Salisbury, UK), was maintained in RPMI medium supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum at 37 °C and 5% CO_2. The OE33 cell line was used at 70% confluency and serum starved in RPMI medium containing 0.5% fetal calf serum for a period of 16 h

before each experiment. For NuGO array nugohs1a520180 microarrays and validations, OE33 cell line was seeded at 50,000 cells per well insert and cultured in a non-contact transwell system (Corning, New York, NY, USA) containing visceral adipose tissue, adipocytes, or control M199 medium for a period of 24 h.

Microarray analysis. RNA was extracted from tumor cell lines using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA purity was assessed and complementary DNA was synthesized using random hexamers (Invitrogen, Carlsbad, CA, USA). Gene expression in the OE33 cell line following non-contact coculture with visceral adipose tissue and adipocytes from viscerally obese EAC patients ($n = 6$) was examined using NuGO array nugohs1a520180 microarrays, custom designed by the Nutrigenomics Organisation (NuGO; www.nugo.org) to include a number of nutrition- and obesity-related genes. We used the 'entrezg' version 14.0.0 annotation from the MBNI custom cdf database, reflecting the latest remapping of Affymetrix probes based on current data in the NCBI database (http://brainarray.mbni.med. umich.edu). All RNA samples processed on Affymetrix microarrays had an RNA integrity number > 6.7 and a 260/280 ratio > 1.65. Following microarray hybridization, all arrays were assessed for quality using a set of standard metrics (including RNA degradation), as implemented in the AffyQCReport library in R;[20] all arrays passed quality control checks. Arrays were then background corrected and normalized using the GCRMA function,[21] and then filtered to remove genes in the lowest 10% of variance using a variance filter. More detailed information on the microarray experiments is available online via the GEO public database (Accession ID: GSE36841).

Quantitative reverse transcriptase PCR. RNA from original array samples and RNA from an independent cohort of cocultured samples was used for array validations, carried out using the following primer sets with dual labeled probes (Metabion, Munich, Germany): PAI-1 (fw: 5′-GCCATGGAACAAGGATGAGATC-3′, rv: 5′-GCCCT GGACCAGCTTCAGA-3′, probe: 5′-Fam-CCACAGACGCGA TCTTCGTCCAGC-Tamra-3′), and primer/probe sets (Applied Biosystems, Carlsbad, CA, USA): SPP1 (Hs00959010_m1), SNAI2 (Hs00161904_m1), and E-cadherin (Hs01023894_m1). For gene expression analysis of tumor biopsies, RNA was extracted from tumor biopsies using Qiagen RNeasy Mini Kit, according to the manufacturer's instructions and analyzed using quantitative reverse transcriptase polymerase chain reaction (qPCR), as above.

Statistical analysis. Analysis of alterations in gene expression in tumor cells following coculture with adipose tissue and correlation between clinicopathological features and gene expression was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). A *P*-value of < 0.05 was taken as a statistically significant association between two variables. Affymetrix microarrays were analyzed using predefined scripts and flexible programming with the R software (www.r-project.org) environ-

Table 1 Patient demographics

	Training cohort	Validation cohort	Patient cohort
No. of patients	6	12	39
Sex (male), *n* (%)	6 (100)	10 (83.3)	39 (100)
Age at surgery, mean (range)	58.2 (49–63)	60.2 (55–69)	64 (48–81)
Diagnosis	EAC	EAC	EAC
BMI (kg/m^2), mean (range)	30.3 (25.4–39)	30 (22–39)	26 (22–39)
WC (cm), mean (range)	107.3 (98–130)	103.8 (81–130)	95.5 (77–130)
VFA (cm^2), mean (range)	209.9 (120.9–297.6)	206 (42.5–383.8)	154 (13–384)
Metabolic syndrome, *n* (%)	2 (33.3)	7 (58.3)	13 (33.3)
Neoadjuvant therapy, *n* (%)	3 (50)	6 (50)	16 (41)

BMI, body mass index; VFA, visceral fat area; WC, waist circumference.

ment for statistical computing. The LIMMA library in R was used to select significantly altered single genes,[22] with a significance threshold of $\alpha = 0.05$ (adjusted for multiple testing). Single-gene changes were ranked by log-fold change (logFC). Gene set enrichment analysis (GSEA) was used to examine the changed pathway expression; the *gsealmPerm* function from the GSEAlm library[23] was modified to test for bidirectional enrichment in KEGG pathways.[24] The association of gene expression on patient survival was determined by Kaplan–Meier survival analysis with gene expression values divided into low- and high-expression groups using the median. Significance was determined using the log-rank (Mantel–Cox) test. Cox regression multivariate analysis was subsequently conducted using forward likelihood ratio on SPSS (IBM, New York, NY, USA). Variables tested on univariate analysis included gene expression, pathological tumor and node stage, lymphatic and hematological dissemination, and tumor differentiation. Variables with a significance level of <0.1 were included in the multivariate model; these were gene expression, pathological tumor and node stage.

Results

Coculture with adipose tissue alters the expression of pro-tumor pathways in an EAC cell line. Differential gene regulation in OE33 cells following coculture with visceral adipose tissue, adipocytes, or control medium was investigated using the NuGO array nugohs1a520180 microarray. Principle component analysis and hierarchical clustering both showed that OE33 cocultured with adipose tissue ($n = 6$) segregated together, separately from OE33 cocultured with adipocytes ($n = 6$) and with control medium ($n = 4$), according to gene expression profile (Figure 1). OE33 cocultured with adipocytes and control medium clustered together, demonstrating that adipocyte coculture had little transcriptomic effect on OE33 cells, in comparison with coculture with whole adipose tissue. Indeed, there were no significant gene alterations in OE33 cells following coculture with adipocytes, indicating that global alterations in gene expression are predominantly mediated not by adipocytes but by secreted factors from the adipose tissue stromal vascular fraction. Pathway analysis showed cytokine signaling, focal adhesion/EMT, and glycolysis to be the top three significantly upregulated pathways in OE33 following coculture with adipose tissue, compared with coculture with

control medium (adjusted $P < 0.05$) (Table 2). The top 10 differentially expressed genes (with adjusted $P < 0.1$) in each pathway were subsequently ranked by logFC; 9 belonging to the glycolysis pathway were listed, as only this number of genes were altered (adjusted $P < 0.1$). There was a total of five genes with a logFC >2; two belonged to cytokine signaling pathways (*CXCL5* and *PPBP*), and three belonged to focal adhesion/EMT pathways (*SPP1*, *PAI-1*, and *SNAI2*) (Table 2). We subsequently investigated altered expression of these latter three genes, established to have direct and important roles in focal adhesion, EMT, and tumor dissemination, in order to elucidate the relationship between obesity and increased cancer mortality. Integrin-binding signaling molecule, secreted phosphoprotein 1 (*SPP1*), underwent a 4.9 log-fold change (adjusted $P < 0.04$), EMT transcription factor, *SNAI2*, underwent a 2.5 log-fold change (adjusted $P < 0.01$) while marker of tumor invasion and metastasis, *PAI-1*, underwent a 2.9 log-fold change (adjusted $P < 0.07$). Gene expression alterations of these transcripts, in addition to laminin-5 subunits, *LAMC2* and *LAMB3*, and integrin subunit $\alpha 3$ (*ITGA3*), were measured by qPCR, in both the training cohort ($n = 6$) and in a larger, independent cohort ($n = 12$), which confirmed the findings of the microarray (see Supplementary Figure 1 online).

Coculture with adipose tissue induces gene expression of *PAI-1* and *SNAI2* in EAC cell lines. Early tumor dissemination is a characteristic hallmark of EAC, contributing to the dismal five-year survival of this aggressive disease. Pathways in tumor invasion and metastasis could therefore present ideal targets for therapeutic intervention. Investigation in a separate cohort ($n = 12$) using qPCR demonstrated that coculture with adipose tissue induced gene expression of *SPP1* by almost 2,000 fold ($P < 0.02$), *PAI-1* by 10 fold ($P < 0.0001$), and the expression of *SNAI2* by over 7 fold ($P < 0.004$) (Figure 2). In order to establish if increased expression of these genes was associated with transition from the epithelial to mesenchymal phenotype, we measured the expression of epithelial cell marker, E-cadherin. We found that coculture with adipose tissue did not alter the expression of SNAI2 target, E-cadherin, in OE33 cells (Figure 2).

Tumor gene expression of members of the EMT pathway, *PAI-1* and *SNAI2*, are increased with obesity, while expression of epithelial cell marker, E-cadherin, is decreased. In order to assess whether tumor EMT is affected by the presence of excess adipose tissue *in vivo*,
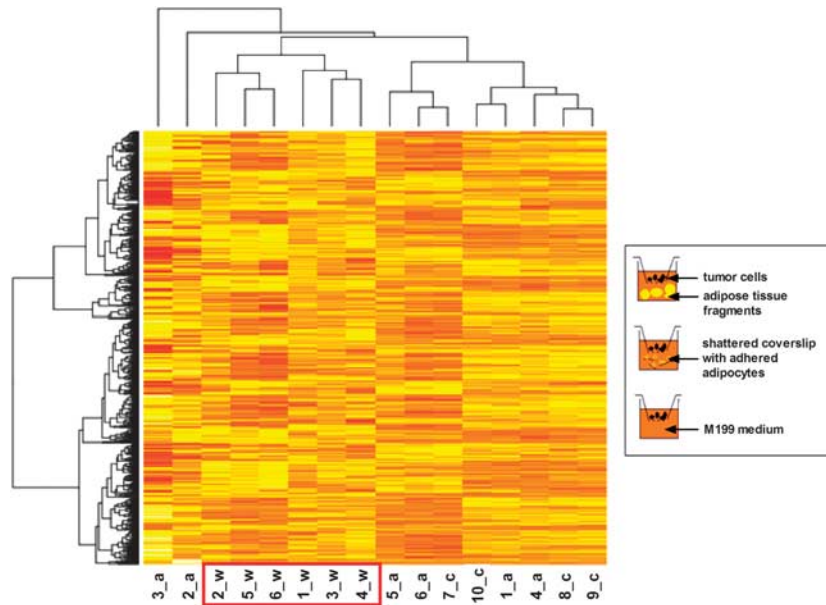
Figure 1 Clustering heatmap analysis of genes differentially expressed in esophageal adenocarcinoma (EAC) cells following coculture with whole adipose tissue, adipocytes, or control medium in the highest 5% of variance identified by Affymetrix microarray analysis. Numbers represent patient number, $w =$ OE33 following coculture with whole adipose tissue, $a =$ OE33 following coculture with adipocytes, and $c =$ OE33 following coculture with control M199 medium.

Table 2 Top differentially expressed genes (ranked by logFC) in top three significantly upregulated pathways in esophageal adenocarcinoma OE33 following coculture with adipose tissue from viscerally obese EAC patients

Pathway	LogFC	Gene	Description
Cytokine signaling*	6.941	CXCL5***	Chemokine (C–X–C motif) ligand 5
	4.370	PPBP	Pro-platelet basic protein (chemokine (C–X–C motif) ligand 7)
	1.871	OSMR**	Oncostatin M receptor
	1.580	SOCS3*	Suppressor of cytokine signaling 3
	1.501	IL1B*	Interleukin 1, beta
	1.082	CCL7*	Chemokine (C–C motif) ligand 7
	0.873	IL4R**	Interleukin 4 receptor
	0.840	IL1R1	Interleukin 1 receptor, type I
	0.424	IL7R*	Interleukin 7 receptor
	0.351	STAT5A	Signal transducer and activator of transcription 5A
Focal adhesion/EMT*	4.919	SPP1*	Secreted phosphoprotein 1
	2.867	SERPINE1	Serpin peptidase inhibitor E1 (plasminogen activator inhibitor-1)
	2.496	SNAI2*	Snail homolog 2
	1.353	LAMB3*	Laminin, beta 3
	1.255	ITGA3**	Integrin, alpha 3
	1.149	LAMC2*	Laminin, gamma 2
	0.656	SERPINB6*	Serpin peptidase inhibitor, B6
	0.504	ITGA6*	Integrin, alpha 6
	0.410	LAMC1	Laminin, gamma 1
	0.269	PXN	Paxillin
Glycolysis*	1.555	PDK4*	Pyruvate dehydrogenase kinase, isozyme 4
	1.406	ALDOC	Aldolase C, fructose-bisphosphate
	1.110	PFKFB3*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
	1.071	HK1**	Hexokinase 1
	1.036	PDK1*	Pyruvate dehydrogenase kinase, isozyme 1
	0.716	PFKP*	Phosphofructokinase, platelet
	0.697	PKM2*	Pyruvate kinase, muscle
	0.589	LDHC	Lactate dehydrogenase C
	0.350	ALDOA	Aldolase A, fructose-bisphosphate

EMT, epithelial mesenchymal transition.
Genes differentially expressed between OE33 following coculture with adipose tissue or control M199 medium (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$).

we compared expression of *SPP1*, *SNAI2*, *PAI-1*, and E-cadherin in tumors of obese and non-obese EAC patients. Gene expression of *SPP1* was not associated with VFA ($P < 0.7$), WC ($P < 0.9$), or BMI ($P < 0.5$), and was therefore dropped from further analysis (data not shown). When patients were grouped by VFA, gene expression
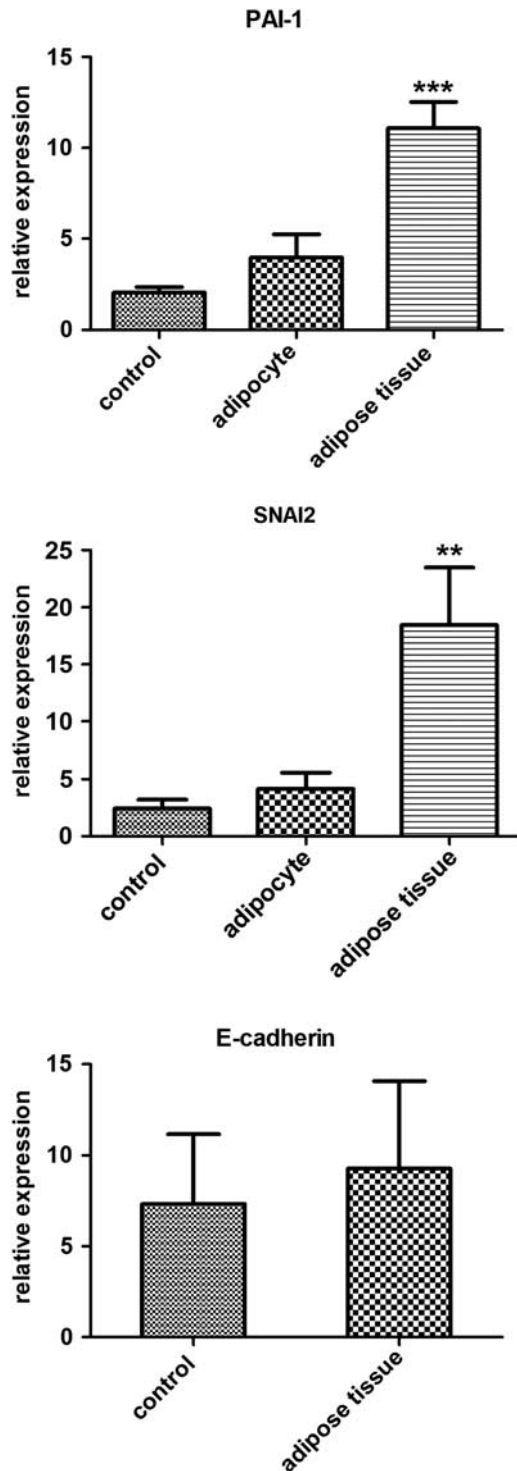
PAI-1

SNAI2

E-cadherin

Figure 2 Coculture of esophageal adenocarcinoma OE33 with visceral adipose tissue induces expression of genes involved in epithelial mesenchymal transition, *PAI*-1 and *SNAI2*. Statistical analysis was performed using paired Student's *t*-test (**$P < 0.01$, ***$P < 0.001$ between control and whole adipose tissue treatment groups).

of *PAI-1* was increased fivefold ($P < 0.05$) in tumors from obese ($n = 26$) relative to non-obese ($n = 13$) EAC patients (Table 3). This significant effect was also observed when

patients were grouped by WC ($P < 0.05$) and by BMI ($P < 0.01$). Gene expression of EMT transcription factor, *SNAI2*, was increased twofold in tumor biopsies from obese relative to non-obese EAC patients. This trend approached statistical significance when patients were grouped by BMI ($P < 0.07$), but not when patients were grouped by measures of visceral obesity, WC ($P < 0.4$), and VFA ($P < 0.3$) (Table 3), indicating that elevated *SNAI2* expression is associated with overall obesity rather than with visceral adiposity. Gene expression of SNAI2 target, E-cadherin, was decreased almost twofold in tumor biopsies from obese relative to non-obese EAC patients. This association reached statistical significance when patients were grouped by WC ($P < 0.05$) and approached significance when patients were grouped by VFA ($P < 0.07$) (Table 3). However, there was no association when patients were grouped by BMI ($P < 0.3$), indicating that, in contrast to *SNAI2*, E-cadherin expression is associated with visceral obesity rather than overall adiposity. There was no association between elevated *SNAI2* expression and reduced E-cadherin expression on pathological tumor or node stage, tumor differentiation, or measures of tumor dissemination. Neither was there any significant association between increased *PAI-1* expression and these markers of aggressive tumor biology; however, a pathologically advanced T stage was accompanied by a twofold increase in *PAI-1* expression ($P < 0.17$) (Table 3). As PAI-1 is elevated in obesity, this trend indicates a potential molecular mechanism by which excess adiposity may be associated with advanced tumor stage.

Elevated *PAI-1* and *SNAI2* expression is associated with poor patient prognosis. EAC is an aggressive disease associated with high patient mortality due to early malignant tumor dissemination.[5] In addition, there is evidence to suggest that obesity is linked to increased cancer mortality.[1] It is therefore of great interest that the gene expression of markers of EMT, *SNAI2*, E-cadherin, and *PAI-1*, was found to be altered in obese EAC patients. Furthermore, using the log-rank (Mantel–Cox) test, we found that elevated *SNAI2* expression was negatively associated with patient survival ($P < 0.05$), identifying this transcriptional regulator of EMT as an obesity-associated prognostic indicator in EAC (Figure 3). Using Cox regression multivariate analysis, we found that *SNAI2* approached significance as an independent prognostic indicator, after adjustment for potential confounders (Hazard ratio: 0.221 (95% CI: 0.047–1.045), $P = 0.057$). Notably, *SNAI2* expression was more strongly associated with EAC survival than established prognostic indicators pathological tumor and node stage ($P = 0.38$, $P = 0.128$, respectively), indicating that our sample size is insufficient to detect a significant association with survival using the Cox regression multivariate analysis method. Elevated tumor levels of these genes in obesity may represent a molecular pathway by which excess adiposity could negatively impact on EAC prognosis. In contrast, decreased expression of SNAI2 target, E-cadherin, showed no association with patient survival. As SNAI2 is an important transcriptional repressor and negative regulator of E-cadherin, a combined survival analysis investigating high *SNAI2* with low E-cadherin was subsequently conducted.

Table 3 *PAI-1*, *SNAI2*, and E-cadherin expression in esophageal adenocarcinoma patients with respect to obesity status and tumor differentiation

	PAI-1 expression, mean (s.e.m.)	*P*	*SNAI2* expression, mean (s.e.m.)	*P*	E-cadherin expression, mean (s.e.m.)	*P*
BMI (kg/m^2)		0.009		0.0693		0.2218
Non-obese <30	1.014 (0.299)		25.623 (3.549)		4.546 (0.847)	
Obese ≥30	3.691 (1.195)		61.411 (24.515)		3.01 (0.656)	
WC (cm)		0.0397		0.3097		0.0447
Non-obese < 94 cm	0.74 (0.164)		26.659 (4.976)		5.423 (1.232)	
Obese ≥94 cm	2.833 (0.808)		46.731 (2.533)		2.999 (0.468)	
VFA (cm^2)		0.031		0.2437		0.0602
Non-obese <130 cm^2	0.529 (0.149)		23.839 (4.134)		5.49 (1.415)	
Obese ≥130 cm^2	2.775 (0.74)		46.655 (14.145)		3.155 (0.442)	
Tumor diff.		0.2782		0.2809		0.5852
Well/moderate	1.517 (0.492)		73.584 (26.049)		4.413 (0.639)	
Poor	2.467 (0.727)		43.901 (9.297)		3.817 (0.865)	
PT		0.1632		0.1796		0.4062
T0–T1	1.569 (0.671)		95.248 (49.316)		3.158 (0.803)	
T2–T3	2.634 (0.748)		48.598 (12.171)		4.301 (0.656)	
PN		0.5241		0.7256		0.2184
N0	1.569 (0.671)		64.717 (28.545)		3.168 (0.569)	
N1	2.166 (0.557)		54.382 (15.01)		4.581 (0.792)	
Lymph inv.		0.3262		0.5117		0.6864
Yes	2.26 (0.606)		55.127 (15.53)		3.90 (0.581)	
No	1.504 (0.561)		77.695 (37.67)		4.45 (1.566)	
Venous inv.		0.4164		0.4169		0.8639
Yes	2.299 (0.606)		48.616 (15.974)		3.963 (0.569)	
No	1.504 (0.561)		72.395 (26.047)		4.161 (1.103)	
Perineural inv.		0.6496		0.4895		0.3857
Yes	2.338 (0.985)		44.257 (13.09)		4.195 (0.949)	
No	1.9 (0.466)		66.371 (20.284)		3.403 (0.433)	

BMI, body mass index; lymph inv., lymph involvement; perineural inv., perineural involvement, expression relative to a calibrator sample (non-obese patient); pN, pathological node stage; pT, pathological tumor stage; tumor diff., tumor differentiation status; venous inv., venous involvement; VFA, visceral fat area; WC, waist circumference.
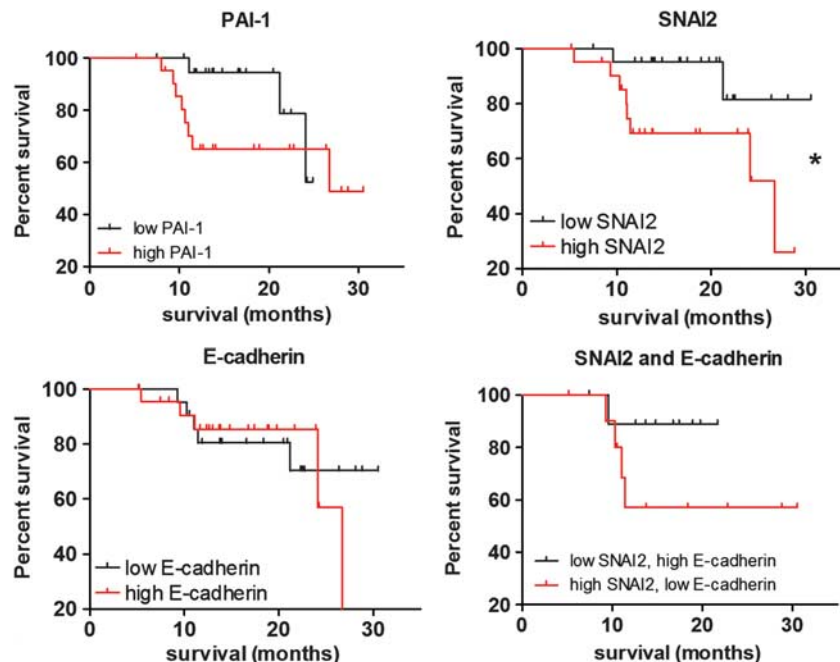

Figure 3 Elevated tumor gene expression of *SNAI2* is associated with poor patient prognosis. Gene expression was separated into above (*n* = 22) and below (*n* = 23) the median expression value, and Kaplan–Meier survival analysis was performed. Statistical analysis was performed using the log-rank (Mantel–Cox) test (*P* < 0.05).

Although no association with survival was found ($P < 0.2$), there was some separation between survival curves following 1 year indicating that it may be worthwhile to reanalyze these data after a longer follow-up period (Figure 3). Alternatively, the mechanism of action of SNAI2 in EAC may be independent of E-cadherin repression; we found no correlation between the expression of transcriptional repressor, *SNAI2*, and expression of its target cell adhesion molecule and epithelial marker, E-cadherin ($r^2 = 0.024$, $P = 0.37$).

DISCUSSION

Esophageal adenocarcinoma (EAC) has one of the strongest epidemiological associations with obesity,[2] although mechanisms remain largely unknown. In this study, we hypothesized that adipose tissue would differentially affect the expression of pro-tumor pathways in EAC cells. To investigate this hypothesis, we used an *ex vivo* explant model to identify altered pathways in EAC cells following coculture with adipose tissue or isolated adipocytes from matched obese EAC patients, relative to control medium. We found that coculture with adipose tissue induced the most pronounced gene alterations in EAC cells, implying that the stromal vascular fraction, comprising a range of cell subsets including endothelial and immune cells, is the primary mediator of this pro-tumor effect.

Using Affymetrix microarrays, we identified glycolysis, cytokine signaling, and focal adhesion/EMT to be the top upregulated pathways in EAC cells following coculture with adipose tissue explants, implicating these pathways in obesity-associated cancer. The reliance of tumor cells on glycolysis even in aerobic conditions is termed the "Warburg effect", a fundamental hallmark of cancer associated with aggressive disease and poor prognosis,[25,26] and adipose tissue-mediated induction of this pathway in tumor cells indicates a mechanism by which obesity promotes cancer mortality. Chronic, low-grade inflammation, a characteristic feature of both obesity[27] and cancer,[28] may have an important role in their epidemiological association. Circulating levels of CXCL5 and IL-1β are elevated in obesity, and exert pro-inflammatory effects linked to the development of insulin resistance and associated pathologies.[29,30] Upregulation of these cytokines in tumor cells by adipose tissue, combined with upregulation of cytokine receptors, oncostatin M receptor, IL-1R, IL-7R, and IL-4R, highlights a mechanism whereby obesity could support pro-inflammatory signaling pathways in cancer. Finally, with tumor metastasis accounting for the majority of cancer-related deaths, and signaling through the focal adhesion pathway necessary for cells to acquire a motile, mesenchymal-like phenotype via the process of EMT,[31] the induction of these pathways in tumor cells by adipose tissue is of profound interest for the elucidation of mechanisms underpinning obesity-associated cancer mortality. We observed a sevenfold increase in expression of EMT transcriptional regulator, *SNAI2*, together with a 10-fold increase in the expression of *PAI-1*, leading us to examine the differential expression of these genes in EAC patients.

Gene expression of *PAI-1* was significantly elevated fivefold in the tumors of obese EAC patients, suggesting a function for this gene in obesity-fuelled cancer. PAI-1 has a role in tumor cell adhesion, and TGF-β-induced PAI-1 expression in OE33 has been demonstrated to correlate with an invasive cell phenotype *in vitro*.[32] Furthermore, PAI-1 is a circulating adipokine, which is elevated in obesity, and high-fat diet-induced obese mice deficient in PAI-1 have reduced adipose tissue mass and increased insulin sensitivity,[33] supporting a potential role for modulation of levels of PAI-1 activity in the treatment of obesity and its comorbidities. We found that *PAI-1* tumor gene expression was elevated in patients with more advanced pathological tumor stage, suggesting a mechanism by which PAI-1 is associated with poor prognosis in breast,[34,35] gastric,[36] rectal,[37,38] and colorectal carcinoma.[39]

There is preliminary evidence that SNAI2, implicated in malignant tumor dissemination via initiation of EMT,[11] may partially exert its functional effect via upregulation of PAI-1 expression, and this has been demonstrated in canine epithelial cells.[8] SNAI2 overexpression is associated with poor prognosis in breast,[40,41] esophageal squamous cell,[42] and colorectal carcinoma,[43] and we have demonstrated it to be a prognostic indicator in EAC. In addition, this study found that gene expression of *SNAI2* was increased twofold, while gene expression of its target, E-cadherin, was decreased twofold, in the tumors of obese EAC patients, implying a role for EMT in obesity-fuelled cancer. Indeed, it has been shown *in vitro* that overexpression of SNAI2 repressed E-cadherin and induced expression of mesenchymal markers vimentin and fibronectin in the OE33 EAC cell line,[44] and that these expression alterations corresponded to a gain in tumor cell proliferation and invasion.[45] Interestingly, coculture with adipose tissue-conditioned medium significantly enhanced EAC cell proliferative, migratory, and invasive capacity *in vitro*,[46] indicating the functional effect of these adipose tissue-induced gene alterations. Increased SNAI2 expression, together with nuclear localization of this transcription factor, has been observed in EAC relative to Barrett's esophagus,[44] indicating the significance of SNAI2 and the process of EMT in the malignant progression of this disease, as well as in EAC tumor invasiveness. SNAI2 overexpression is not always associated with E-cadherin repression, and this has been demonstrated in breast[40,41] and esophageal squamous cell carcinoma.[47] We report that gene expression levels of E-cadherin were not associated with prognosis, indicating that E-cadherin-independent pathways may be important in SNAI2-mediated pathogenesis in EAC.

In summary, we have demonstrated a novel association of *PAI-1* and *SNAI2* with obesity and of *SNAI2* with prognosis in EAC, highlighting a potential mechanism by which excess adiposity may promote malignant tumor dissemination and contribute to the poor prognosis characteristic of this aggressive disease. Although not yet in clinical trials, small molecule PAI-1 inhibitors are being developed as anti-thrombotic agents and are currently under investigation in animal models of pulmonary fibrosis;[48] this study indicates that it would be of interest to examine the effect of these PAI-1 inhibitors in the context of obesity-fuelled cancer. In conclusion, this work has identified PAI-1 and SNAI2 as additional

candidates for the development of therapeutic strategies targeting pathways of tumor dissemination in EAC, with the ultimate goal of stratified treatment of obese EAC patients.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✔ Esophageal adenocarcinoma (EAC) is a lethal disease, increasing in prevalence.
- ✔ There is a strong epidemiological association between obesity and EAC, with over 40% of cases linked to excess adiposity.
- ✔ Molecular mechanisms underlying this association are largely unknown.

WHAT IS NEW HERE

- ✔ Visceral adipose tissue upregulates pro-tumor pathways in EAC.
- ✔ Expression of epithelial mesenchymal transition promoters, *PAI-1* and *SNAI2*, is elevated in tumors of viscerally obese EAC patients.
- ✔ Elevated tumor *SNAI2* expression is associated with poor survival, indicating a potential mechanism whereby obesity may contribute to increased mortality.

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Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)