**OPCML is hypermethylated in a subset of patients with metaplastic changes in their esophagus**

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**Abstract**

OPCML hypermethylation is considered a promising cancer biomarker. We examined methylation levels in the first exon of OPCML in two patient cohorts within the esophageal adenocarcinoma and gastric adenocarcinoma cascades and in a range of cell-lines using a custom PyroMark CpG assay. Methylation levels were significantly higher in esophageal tissue with histologically confirmed glandular mucosa as compared to tissue from normal esophagi or gastro-esophageal reflux disease. Higher levels of OPCML methylation were absent in the adjacent normal esophageal tissue of patients with glandular mucosa. Higher levels of methylation were confirmed in cell-lines derived from patients with adenocarcinoma, but also detected in two cell-lines with signs of dysplasia. We validated our assay by showing no differences in methylation levels in DNA extracted from blood of patients within the gastric adenocarcinoma cascade.

**Keywords:** OPCML, CpG methylation, Dysplasia, Adenocarcinoma

**Introduction**

OPCML (Opioid Binding Protein/Cell Adhesion Molecule Like) belongs to the IgLON glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecule family. IgLONs are highly conserved proteins that can anchor to the cell membrane surface by their hydrophobic tails [1, 2]. The expressed OPCML protein follows these patterns where it is localized in the plasma membrane and is highly conserved [3]. Although levels of expression vary, studies have demonstrated OPCML expression in many tissue types, including brain, ovary, heart, placenta, testes, kidney, liver, spleen, pancreas, colon, cervix, prostate, trachea and stomach [1, 2, 4–6], with weak expression in the lung, breast and bone marrow [1].

The CpG island of OPCML has been shown to be methylated at relatively low levels in healthy tissue [7]. In contrast, OPCML hypermethylation was first associated with epithelial ovarian cancer [7]. This inactivation was later associated with hepatocellular carcinoma [8], lung adenocarcinoma [9] and gastric [10] and brain cancers [2], followed by a variety of other cancers from both primary and metastatic tumors as well as tumor cell lines, including those derived from esophageal adenocarcinoma (EAC) [1]. The hypermethylation of OPCML and its consequential transcriptional silencing in a wide variety of cancers is indicative of its role as a tumor suppressor gene (TSG) as well as its role as a potential biomarker for cancer [1, 11]. Further, induced expression in vitro has been found to inhibit the growth of cancer cell lines. Transfection of gastric cancer cell-lines SGC-7901 and BGC-823 with OPCML-pcDNA3.1 plasmid resulted in accumulation of cells in the G0/G1 phase and induction of apoptosis [5], suggesting it may also be a therapeutic target.

It has been proposed that OPCML acts as a TSG by interacting with other IgLONs, and loss of this function results in reduced cell adhesion and impairment of...
signalling between IgLONs [1]. However, McKie et al have shown that OPCML binds the extracellular domains of receptor tyrosine kinases (RTKs), altering their levels, trafficking and downstream signalling, and thus, leading to the inhibition of cellular growth [12].

Here, we examined the methylation levels of OPCML locally and systemically in the inflammatory cascades leading to upper gastrointestinal adenocarcinomas.

**Materials and methods**

**Recruitment of patients within the esophageal adenocarcinoma cascade**

One hundred fifty two predominantly Caucasian subjects who underwent upper gastrointestinal endoscopy at the Prince of Wales Hospital (Sydney) for examination of their gastrointestinal symptoms were recruited prospectively. Subjects who had been prescribed antibiotics or non-steroidal anti-inflammatory drugs in the two-month period prior to recruitment were excluded. A set of two samples were collected at endoscopy, one sample for assessment of OPCML methylation locally (esophageal tissue) and another from the same location for histological analysis grouped patients into four groups (normal esophagus, GERD, n = 42; GM, n = 20; EAC, n = 1). In a subset of patients (GM, n = 11), additional samples histologically classified as normal from regions adjacent to the GM region were collected. Further, three samples (normal, GM, and EAC regions) were collected from one patient diagnosed with EAC. All samples were snap-frozen in cryotubes following collection and stored at −80 °C until processing. Ethics approval was obtained from the South Eastern Sydney Local Health District Human Research Ethics Committee (HREC 13/375 and HREC 16/020). All subjects recruited to the study signed a written informed consent, and all experiments were performed in accordance with relevant guidelines and regulations.

**Recruitment of patients within Correa’s (gastric adenocarcinoma) cascade**

The gastric adenocarcinoma (GAC) cascade study population comprises Mestizo individuals (54 non-cardia GAC cases and 55 age-matched functional dyspepsia controls) from Pereira, a city located in the Colombian Andes mountain range, who underwent upper gastrointestinal endoscopy at Comfamiliar Risaralda Hospital. Patients known to be infected with the Human Immunodeficiency Virus, who have any comorbidity associated with immunosuppression or who had been prescribed non-steroidal anti-inflammatory drugs, anti-microbial agents or acid suppressants in the three-month period prior to recruitment were excluded. As Colombian individuals present with GAC early in life, subjects >30 years were recruited, the critical age from which the incidence increases steadily thereafter in this population. In addition, 91 subjects with gastric precancerous lesions composed of 40 subjects with atrophic gastritis, 40 subjects with intestinal metaplasia and11 subjects with dysplasia, based on histological assessment by experienced pathologists, have been included. In addition to gastric biopsies for histological assessment, a blood sample for assessment of OPCML methylation systemically was collected at endoscopy. Blood samples (5 ml) were collected intravenously in BD Vacutainer™ SST™ Blood Collection Tubes and immediately stored at −20 °C until they were shipped frozen on dry ice to Australia. Samples were then stored at −20 °C until processing. Ethics approvals have been gained from Comfamiliar Risaralda Hospital (Acta 5140) and the University of New South Wales (UNSW) (HREC 16010) human research ethics committees.

**Growth and maintenance of human cell lines**

HET-1A cells were grown in 10 ml cell culture media comprising LHC-9 media (Catalog no. 12680013, ThermoFisher Scientific; Scoresby, Vic, Australia) supplemented with 10% heat-inactivated fetal bovine serum (hi-FBS) and 100 μg/ml penicillin/streptomycin, in 25 cm² tissue culture flasks at 37 °C with 5% CO₂. CP-A, CP-B, CP-C, and CP-D were grown in Keratinocyte-SFM media (Catalog no. 17005042, ThermoFisher Scientific) supplemented with prequalified human recombinant Epidermal Growth Factor 1–53 (5 μg/l), Bovine Pituitary Extract (50 mg/l), 10% hi-FBS and 100 μg/ml penicillin/streptomycin. OE33, AGS, and LS 174 T cells were grown in Roswell Park Memorial Institute (RPMI) media supplemented with 10% hi-FBS and 100 μg/ml penicillin/streptomycin. Caco-2 cells were grown in Minimum Essential Medium supplemented with 10% hi-FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2.25 mg/l sodium bicarbonate and 100 μg/ml penicillin/streptomycin.

**DNA extraction**

Our recruitment of the two study cohorts resulted in two types of samples: 1) mucosal samples from the esophagus of subjects recruited to the EAC cascade cohort; 2) blood samples collected intravenously from subjects recruited to the GAC cascade cohort. DNA was extracted from esophageal samples and human cell-lines using Gentra Puregene Tissue kit (Qiagen; Chadstone Centre, Vic, Australia) according to the manufacturer’s instructions. DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer’s instructions.

**Custom OPCML methylation assay**

The custom OPCML methylation assay was performed using services and facilities available at the Australian
the PyroMark Q24 software to give the methylation
the manufacturer’s instructions. Data was analyzed on
the PyroMark Q24 software to give the methylation
values (%) for each CpG site in the sample.

Statistics
Comparisons of OPCML methylation levels across pa-
tient groups were performed using one-way ANOVA
with post hoc Tukey’s test and false discovery rate cor-
rection where applicable.

Results
OPCML methylation in esophageal samples of patients
within the esophageal adenocarcinoma cascade
Methylation levels of three CpG sites within exon 1 of
OPCML (position 1: hg19_chr11:133402185; CpG site:
cg19151121) were measured in esophageal samples from
patients within the EAC cascade. We observed signifi-
cantly higher levels of methylation across the three posi-
tions in patients that had histologically detectable
glandular mucosa (GM) as compared to patients with a
normal esophagus and those with gastro-esophageal re-
flux disease (GERD) (Fig. 1a-c). However, on further
analysis, it was clear that there was no consistent methy-
lization pattern across the GM group (Fig. 1d), and the
significant differences with respect to normal and GERD
patients were driven by intermediate to high methylation
patterns in a specific subgroup of GM patients.

Importantly, of the 20 patients with GM, normal
esophageal tissue samples (confirmed histologically)
from 11 of the patients were collected from regions adja-
cent to the GM region. No hypermethylation was ob-
served in the normal tissue from these patients (Fig. 1e).

As part of the prospective enrolment of subjects, one
EAC patient was recruited. During endoscopy, the patient
was visually suspected to have an EAC, and thus, three
samples were collected from regions that were later histo-
logical confirmed as normal, GM and EAC. OPCML
methylation was confirmed to show a step-wise increase
across the three sites and three positions (Fig. 1f).

OPCML methylation in human cell-lines
Given the signature observed in the esophageal sam-
ple from patients within the EAC cascade, we then
measured methylation levels of the same three sites
within OPCML in nine esophageal, gastric and intesti-
nal cell-lines. Methylation patterns were consistent
across all three positions (Fig. 2a-c). Cell-lines derived
from upper gastrointestinal adenocarcinomas (OE33
and AGS) were methylated higher as compared to
those derived from lower gastrointestinal adenocarcin-
omas (LS 174 T and Caco-2; Fig. 2). Despite the fact
that EAC cascade cell-lines CP-B, CP-C and CP-D
are derived from areas of high grade dysplasia while
CP-A is derived from an area of nondysplastic meta-
plasia [13, 14], we observed low levels of methylation
for CP-A, CP-B and CP-C when compared to CP-D
(Fig. 2). In fact, CP-D showed similar methylation
levels to the EAC cell-line OE33. Surprisingly, we ob-
served intermediate levels of OPCML methylation in
immortalized primary normal esophageal cells
HET-1A (Fig. 2).
OPCML methylation in blood of patients within the gastric adenocarcinoma cascade

Given that peripheral blood mononuclear cells (PBMCs) do not express OPCML [1] and OPCML is hypermethylated in GAC tissue [10], we then recruited a cohort of patients across the GAC cascade (n = 200) and collected blood intravenously. This was performed to validate our PyroMark assay and local tissue results in a large cohort of patients within an inflammatory adenocarcinoma cascade employing samples (i.e. blood) that should not show elevated methylation levels of OPCML.

OPCML methylation was measured in DNA extracted from blood samples (Fig. 3).

Discussion

OPCML promoter epigenetic silencing in the form of hypermethylation has been proposed as a potential biomarker of a range of cancers [1, 7–11]. Here, we show that intermediate levels of OPCML exon 1 methylation in the local tissue occurs in a subset of individuals with metaplasia.

Patients within the EAC cascade were recruited and OPCML methylation levels were examined in esophageal tissue samples. Significant differences in OPCML methylation patterns were observed between patients with GM as compared to patients with normal esophagi and GERD. These differences were found to be driven by intermediate and high methylation patterns within individual patients rather than a consistent methylation pattern across the entire GM subgroup. Further, hypermethylation was not detected in normal esophageal tissue from patients with GM. We suggest that OPCML hypermethylation in these patients could suggest early signs of dysplastic events not detected histologically. While arising from only one patient, and thus, should be assessed with caution, the importance of OPCML hypermethylation is supported by our comparisons across the EAC histological cascade within one EAC patient.

In esophageal epithelial cell-lines across the EAC cascade, high levels of methylation were observed in one of the three cell-lines derived from dysplastic Barrett’s esophagus (BE) patients as well as in the cell-line derived from an EAC patient. Methylation levels similar to those in normal tissue were observed in the cell-line derived from non-dysplastic BE. Intriguingly, HET-1A, an immortalized esophageal epithelial cell-line derived from normal squamous tissue showed intermediate levels of

**Fig. 1** OPCML methylation in normal esophageal samples or from patients within the EAC cascade. Position 1 (a), position 2 (b), and position 3 (c) within the first exon of OPCML. d OPCML methylation at position 1 across the 20 patients with GM. Site of GM was analyzed. Position 1 was shown as representative results across the three positions. e OPCML methylation at position 1 across the 11 patients with GM. Both normal and GM tissue were analyzed from the same patient. Patients corresponded to P1–11 in Panel D. Patterns were consistent for positions 2 and 3. f OPCML methylation at positions 1, 2 and 3 for the normal, GM and EAC tissues within the same EAC patient (n = 3 replicates across all measurements). Position 1: hg19_chr11:133402185; CpG site: cg19151121. *: P < 0.05; **: P < 0.01; ***: P < 0.001. P: Patient; GERD: gastro-esophageal reflux disease; GM: detectable glandular mucosa; EAC: esophageal adenocarcinoma
OPCML methylation. This is highly relevant considering Underwood et al. have shown HET-1A cells appear dysplastic and fail to display evidence of squamous differentiation [15]. This data would further suggest that
OPCML hypermethylation is associated with specific dysplastic events in esophageal tissue, and HET-1A will be a useful cell-line to delineate the events that lead to OPCML hypermethylation.

The assay in this study was designed to target three sites within the first exon of OPCML for two reasons. First, we set out to design a rapid assay that can be translated into clinical applications in the future. Second, hypermethylation of the first exon of genes has been reported to be more tightly linked to transcriptional silencing than methylation in the promoter region [16]. To validate our assay and local tissue results, a large cohort of patients within the GAC cascade was recruited and blood collected. No hypermethylation of OPCML was observed in any of the disease groups, and this is consistent with the lack of expression of OPCML in PBMCs [1]. The assay also showed high consistency across the 200 subjects within this cohort. Given that hypermethylation of the OPCML promoter has been linked to downregulation of expression [5], further validation of the effects of hypermethylation of OPCML exon 1 should come in the form of OPCML expression profiling in local tissues. Moreover, there is a need to determine if OPCML has similar effects on receptor tyrosine kinases in the EAC cascade as has been observed in ovarian cancer [12, 17].

In conclusion, OPCML hypermethylation appears to be a signature that should be investigated further in carcinogenesis. Additional studies assessing larger cohorts are required to confirm the importance of OPCML methylation prior to tumor development.

Abbreviations
BE: Barrett’s esophagus; CpG: Cytosine-phosphate-guanine; EAC: Esophageal adenocarcinoma; GAC: Gastric adenocarcinoma; GERD: Gastro-esophageal reflux disease; GM: Glandular mucosa; GP: Glycosylphosphatidylinositol; HREC: Human Research Ethics Committee; OPCML: Opioid Binding Protein/Cell Adhesion Molecule Like; RTK: Receptor tyrosine kinase; TSG: Tumor suppressor gene

Acknowledgements
The authors would like to thank Dr. David Chandler, Australian Genome Research Facility, for his help with the OPCML methylation assay.

Funding
NOK is supported by a Cancer Institute NSW Career Development Fellowship (15/CDF/1–11). NCR is supported by a National Health and Medical Research Council, Australia Early Career fellowship (APP1111461) and Cancer Australia priority-driven collaborative cancer research grant (1129488).

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors’ contributions
NOK, NCR, SMR contributed reagents and funds. SMR and NOK recruited the EAC cascade cohort. NCR, GLPH, JLCD, AJVC, HWTH, ARCA, OzshR recruited the GAC cascade cohort. NCR, GLP and NOK performed the DNA extractions. NOK performed the statistical analyses. NOK drafted the manuscript. NCR, GLP, GLPH, JLCD, AJVC, HWTH, ARCA, OzshR, SMR, NOK read, corrected and approved the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Esophageal adenocarcinoma cohort samples: Ethics approval was obtained from the South Eastern Sydney Local Health District Human Research Ethics Committee (HREC 13/375 and HREC 16/020). Gastric adenocarcinoma cohort samples: Ethics approvals have been gained from Comfmarinal Risarada Hospital (Acta S140) and the University of New South Wales (UNSW) (HREC 16010) human research ethics committees. All subjects recruited to the study signed a written informed consent, and all experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 1 October 2018 Accepted: 28 November 2018
Published online: 07 December 2018

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