Accepted Preprint first posted on 3 January 2017 as Manuscript ERC-16-0419

1 **Progressive epigenetic dysregulation in neuroendocrine tumour liver metastasis.**

2 Anna Karpathakis[1,3], Harpreet Dibra[1], Christodoulos Pipinikas[1], Andrew Feber[1], Tiffany
3 Morris[1], Joshua Francis[2], Dahmane Oukrif[1], Dalvinder Mandair[1,3], Marinos Pericleous[3],
4 Mullan Mohmaduvesh[3], Stefano Serra[4], Olagunju Ogunbiyi[3], Marco Novelli[1], TuVinh
5 Luong[3], Sylvia L Asa[4], Matthew Kulke[5], Christos Toumpanakis[3], Tim Meyer[1,3], Martyn
6 Caplin[3], Stephan Beck[1], Christina Thirlwell[1,3].

7 Affiliations: [1]University College London, London, UK; [2]The Broad Institute, Boston, USA;
8 [3]The Royal Free Hospital, London, UK; [4]UHN Princess Margaret Cancer Centre, Toronto,
9 Canada; [5]DanaFaber Cancer Institute, Boston, USA

10 Corresponding author: Dr Christina Thirlwell, UCL Cancer Institute, 72 Huntley St, London,
11 WC1E6BT. Tel: 020 76796882, Fax: 020 76796817, christina.thirlwell@ucl.ac.uk

12 Keywords: Neuroendocrine tumour, epigenetics, methylation, metastasis

13 Short Title: Epigenetics of SINET liver metastases

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15 Word count: 1494

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Dear Editor

The incidence of small intestinal neuroendocrine tumours (SINETs) is increasing and distant metastases are present at diagnosis in 70% of cases, the liver being the commonest site of metastasis (*Yao et al. 2008*). Despite this, our understanding of the mechanisms underlying metastatic progression of SINETs is currently limited and prior studies of the molecular biology of SINET liver metastases (LM) have been performed predominantly in small cohorts utilising candidate based techniques.

SINETs have a low rate of mutations compared to most cancers. Tthe most frequently mutated gene is *CDKN1B* (encoding p27, a cell cycle regulator); however mutations in this gene occur in only 8% of tumours and there is no characteristic mutational hotspot (*Francis et al. 2013)*. Furthermore, mutation of CDKN1B does not correlate with expression of p27 (*Crona et al. 2015)*. We previously identified that SINETs are epigenetically dysregulated, and a panel of candidate driver epimutation genes has been identified *(Karpathakis et al. 2016)*. Therefore we postulated that metastatic progression in SINETs may also be epigenetically regulated. Here we present findings from the largest molecular profiling study of SINET LM performed to date, integrating copy number variance (CNV), DNA methylation and RNA expression profiling to characterise the mechanisms underlying metastatic progression.

Experimental details of DNA methylation, CNV and RNA expression profiling are as previously published *(Karpathakis et al. 2016)*. Patients provided informed consent for their tissue to be analysed in this study which was Research Ethics Committee approved (Ref: 09/H0722/27). All cases were reviewed by two expert NET histopathologists (TVL/MN). Nucleic acids were extracted using standard methods (Qiagen:QIAamp DNA Mini kit, Roche:High Pure RNA Paraffin kit). H&E stained sections were evaluated to ensure >80%

46 purity of tumour specimens. Methylation profiling was performed on the
47 HumanMethylation450 BeadChip (HM450)(Illumina). Methylation data analysis was
48 performed using ChAMP pipeline
49 (https://www.bioconductor.org/packages/release/bioc/html/ChAMP.html). Whole genome
50 methylation profiling using Methylated DNA Immunoprecipitaion sequencing (MeDIP) was
51 performed as previously described. MeDIP data was analysed using the custom pipeline
52 MeDUSAv2.0 (https://www.ucl.ac.uk/cancer/research/department-cancer-biology/medical-
53 genomics-group/past-projects/medusa-project). Gene expression analysis was performed on
54 the Whole genome cDNA-mediated annealing, selection and ligation (DASL)(Illumina)
55 assay. Expression data was analysed using the 'limma' package in R
56 (https://bioconductor.org/packages/release/bioc/html/limma.html). Raw data from this study
57 will be deposited in GEO (Accession number: XXXXXX)

58 In summary, n=90 samples underwent array based DNA methylation analysis, n=26 samples
59 underwent methylation specific immunoprecipitation followed by DNA sequencing, and
60 n=49 underwent array based RNA expression analysis. Of cases with relevant clinical data,
61 93% had received no systemic treatment prior to specimen collection (27/29 cases).

62 The CNV profile of SINET LM (n=20) mirrors that of primary tumours with the most
63 frequent alteration of chr18 LOH seen in 79% of cases. A greater proportion of LM
64 demonstrate amplification of chr20 (42%), deletion of chr19 (35%), whilst gain of 17q is
65 found only in LM (21%). A trend of increased incidence of CNVs was seen in LM compared
66 to SINET primary tumours (SINET primary: median 78megabasepair; LM: median 114mbp,
67 p=0.08).

68 Comparison of methylation profiles of SINET LM to that of primary SINETs identified
69 29,263 methylation variable positions (MVPs) (adj p <0.05). Using a cut off of >30%

70 difference in methylation between SINET primaries and LM, MVPs involving eight genes
71 were identified (*CLEC16A, HOXC4, HOXD4, IGF2AS, INS-IGF2, LDHA, RTN4RL1,*
72 *SASH1)*. This suggests that the methylation profile of SINET primaries and metastases are
73 broadly similar and that these epigenetic differences occurring in metastatic progression may
74 be more subtle than those involved in primary tumorigenesis. Global hypomethylation is
75 noted in SINET primary tumours and occurs to an even greater extent in SINET LM (normal
76 tissue methylation 0.628, primary 0.572, LM 0.515, $p<0.001$).

77 Almost three thousand (n=2857) genes were significantly differentially expressed between
78 LM and primary tumours. Using a > 3-fold alteration in gene expression, more genes were
79 found to be upregulated (n=321) than downregulated (n=171) in LM. KEGG pathway
80 analysis (http://bioinfo.vanderbilt.edu/webgestalt/) of differentially expressed genes between
81 LM and SINET primary identified significant enrichment of multiple cancer related pathways
82 overexpressed in LM including PI3K signalling events, ErbB1 downstream signalling,
83 PDGFRβ signalling pathway, and mTOR signalling pathway (adjusted $p<0.001$).

84 Analysis of SINET LM identified progressive changes between SINET primaries and LM in
85 DNA methylation and RNA expression in genes which had previously been identified in
86 primary tumours when compared to normal tissue. This phenomenon was observed in a panel
87 of 21 epigenetically dysregulated candidate driver genes which was previously identified
88 *(Karpathakis et al. 2016)*(**Table 1**).

89 LM demonstrated hyper/hypomethylation of all 21 genes in concordance with the pattern
90 seen in SINET primaries when compared to normal tissue. In 19 genes (90.5% of the panel)
91 a trend for progressive hyper/hypomethylation was demonstrated in LM, of which 14 (66.6%)
92 of the panel were significantly differentially methylated compared to the primary SINET.

93 All of the 21 genes included in the panel demonstrated over/under expression in LM in
94 concordance with SINET primary tumours. In 15 genes (71.4% of panel) there was a
95 progression of aberrant expression to a greater extent in LM than was demonstrated in SINET
96 primaries (**Figure 1, Table 1**).

97 Validation of SINET LM methylation status was performed in an independent cohort of
98 seven LM profiled by methylated DNA immunoprecipitation sequencing (MeDIP-seq). It
99 was demonstrated that 20/21 (95.2%) genes exhibited concordant trends in methylation
100 during progression from NSI to SI primary tumour to liver metastasis as was identified using
101 HM450 profiling. Statistically significant progressive aberrant methylation was
102 demonstrated in 10/21 genes (47.6%).

103 Validation of SINET LM expression status was performed utilising a publicly available
104 dataset including three SINET primaries and three LM (GSE9576, Leja *et al.* 2009). In total,
105 10/21 (47.6%) of the candidate panel demonstrated a trend for progressive dysregulation in
106 LM compared to primary tumours in keeping with the findings from the discovery dataset.
107 The small number of cases included in this validation set limit the ability to confirm
108 statistical significance.

109 Through integrated DNA methylation, CNV and RNA expression analysis we have identified
110 progressive genomic derangements in SINET LM when compared to primary tumours.

111 CNVs were seen more frequently in LM, in particular arm level amplifications, as previously
112 reported (*Hashemi et al. 2013)*. Amplification of chromosome 17q was observed more
113 frequently in LM than primaries in this cohort. This alteration has previously been described
114 in both SINET and pancreatic NET primary tumours but this is the first time that increased
115 frequency in LM has been identified. Chromosome 17 harbours the proto-oncogene
116 HER2/neu (17q11-21), amplification of which may be related to a more aggressive
117 phenotype.

118 The finding of progressive global hypomethylation in metastases compared to primary
119 tumours is in keeping with previously published data (*Verdugo et al. 2014).* A pattern of
120 progressive aberrant methylation observed in our previously identified panel of 21
121 epimutated genes in liver metastases, suggests that increasing epigenetic dysregulation may
122 drive progression to metastasis.

123 Transcriptome profiling demonstrated differential expression of 492 genes between LM and
124 SINET primary tumours which may represent drivers of metastasis, including components of
125 the PI3K/mTOR pathways. Progressively dysregulated expression of the panel of candidate
126 genes was demonstrated in liver metastases compared to primary tumours. This indicates
127 escalating deregulation of aberrant expression of the genes and pathways associated
128 development of SINET primary tumours occurs in association with metastatic progression.

129 In total, 71.4% (15/21, expression) to 90.5% (19/21, methylation) of cases are affected by
130 progressive dysregulation in association with metastasis. The gene encoding the gastric
131 inhibitory polypeptide receptor (*GIPR*) is one of a panel of epigenetically dysregulated genes
132 in SINETs which is significantly progressively hypermethylated in LM compared to primary

133 tumours. This may represent a target for novel therapeutic agents in the management of
134 SINETs, and has already been investigated as a target for novel imaging modalities (*Sherman*
135 *et al. 2013)*.

136 In summary, integrated genomic analysis of a large cohort of SINET LM has identified novel
137 molecular mechanisms associated with metastatic progression. Epigenetic dysregulation of a
138 panel of 21 candidate genes was identified in LM concordant with those found in primary
139 SINET. Components of cancer related pathways including PI3K, mTOR and ErbB1 are
140 overexpressed in liver metastases compared to normal tissue, which may be utilised as
141 therapeutic targets. Current clinical practice includes the use of agents targeting the mTOR
142 pathway is based on evidence from the RADIANT trials of everolimus in pancreatic and
143 SINETs (*Yao et al. 2016)*. The use of second line dual mTORC/PI3K inhibition for
144 pancreatic NETs was not supported in a recently clinical trial (*Fazio et al. 2016)*. Our data
145 suggest the development of novel agents targeting epigenetic modifications in these pathways
146 may hinder metastatic progression.

147 Large scale alterations in the transcriptome of SINET LM compared to primary tumours have
148 been identified, with more subtle alterations in the methylome. This may indicate that small
149 alterations in the epigenetic status of key genes are sufficient to drive metastatic progression,
150 or that alternative mechanisms are also contributing to progression including for example
151 histone modifications. To date there have been no identified driver genetic mutations
152 responsible for SINET development or progression. The data presented in this manuscript
153 suggest that epigenetic alterations are significant in this tumour type. We believe that future
154 research should be focused on further elucidating epigenetic mechanisms in the evolution of
155 neuroendocrine tumours.

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Acknowledgments

This work was supported by the UCL Experimental Cancer Medicine Centre and the NIHR UCLH Biomedical Research Centre

Disclosure

I declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure and Table Legends

Figure 1 Methylation and expression profile of SINET LM demonstrates progressive dysregulation compared to SINET primary tumours in a panel of genes

Table 1 Methylation and expression profile of SINET LM demonstrates progressive dysregulation compared to SINET primary tumours in a panel of genes. *Normal small intestine (NSI), Small intestinal primary neuroendocrine tumour (SINET), Liver metastasis (LM).*

	Median methylation					Median Expression				
	NSI	SINET	LM	Lm progressing trend?	p (LM vs SINET)	NSI	SINET	LM	Lm progressing trend?	p (LM vs SINET)
Downregulated :										
CDX1	0.55	0.90	0.91	yes	0.48	5,481.4	1,503.0	570.8	yes	**0.03**
FBP1	0.43	0.82	0.83	yes	0.58	10,896.0	2,193.1	2,937.5	no	0.54
TMEM171	0.18	0.61	0.73	yes	0.11	3,632.7	519.6	201.9	yes	0.08
C20orf54	0.34	0.64	0.60	no	0.64	2,320.5	577.0	176.7	yes	0.056
GATA5	0.89	0.69	0.57	yes	**0.006**	658.2	64.0	65.8	no	0.96
NGEF	0.89	0.72	0.60	yes	**0.018**	3,819.7	512.2	309.9	yes	0.23
PNLIPRP2	0.77	0.47	0.33	yes	**0.007**	1,419.4	235.0	95.5	yes	0.34
TRIM15	0.53	0.87	0.87	no	0.65	615.8	93.1	27.2	yes	**0.009**
Upregulated :										
PTPRN	0.38	0.11	0.07	yes	**0.001**	349.8	4,319.1	5,456.3	yes	0.1
C3orf14	0.32	0.12	0.07	yes	**<0.001**	212.4	1,016.7	1,803.9	yes	**0.002**
CNTNAP5	0.26	0.09	0.08	yes	**0.009**	280.3	4,343.1	3,518.3	no	0.22
DSCAM	0.50	0.15	0.08	yes	**<0.001**	59.9	1,142.3	1,028.6	no	0.61
GDAP1L1	0.38	0.08	0.05	yes	**0.002**	74.3	659.7	1,051.8	yes	0.07
PCSK1	0.38	0.08	0.05	yes	**0.001**	217.9	2,420.4	2,911.0	yes	0.39
PRLHR	0.44	0.13	0.06	yes	**<0.001**	193.6	3,909.3	2,676.2	no	0.08
SNTG1	0.28	0.07	0.04	yes	**<0.001**	78.6	2,126.9	3,396.3	yes	**0.003**
CELSR3	0.10	0.57	0.67	yes	0.11	2,029.6	7,869.0	9,442.6	yes	0.17
GIPR	0.30	0.66	0.74	yes	**0.034**	248.1	1,900.0	2,283.6	yes	0.27
KCNH6	0.07	0.36	0.48	yes	**0.01**	998.3	5,146.7	5,935.3	yes	0.35
LMX1B	0.30	0.59	0.60	yes	0.99	286.8	3,503.9	1,979.4	no	**0.034**
RUNDC3A	0.08	0.42	0.66	yes	**0.02**	454.2	3,966.4	5,677.8	yes	0.081

Table 1

Methylation

Expression