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Potential molecular mechanism of AIP-mediated cellular invasion

S Barry[1], Eivind Carlsen[3]
J A Saleh[1], E Gadaleta[2], C Chelala[2] and M Korbonits[1]
[1]Department of Endocrinology, William Harvey Research Institute
[2]Centre for Molecular Oncology, Barts Cancer Institute,
[3] Department of Pathology, STHF, N-3710 Skien, Norway
Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, UK

1. Introduction

Heterozygote germline mutations in the aryl-hydrocarbon receptor interacting protein (*AIP*) gene play a role in the pathogenesis of pituitary adenoma development in familial isolated pituitary adenoma (FIPA) as well as simplex pituitary adenoma cases. *AIP* mutation positive patients develop often aggressively growing tumours in early teenage years and often show invasion at the time of diagnosis as well as poor response to somatostatin analogues than sporadic tumours[1,2].

2. Aims

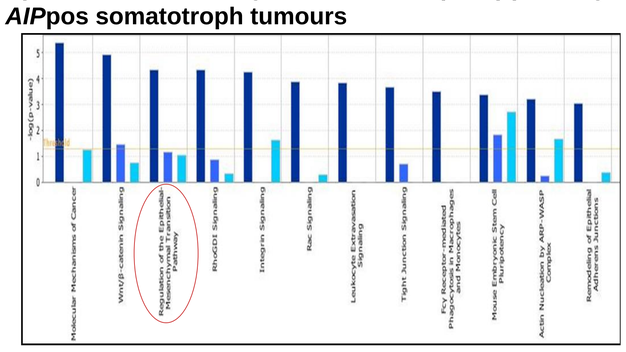
The aim of this study was to perform comparative gene expression analysis of *AIP* mutation-positive (*AIP*pos) pituitary adenomas to discover the genes/pathways responsible for the aggressive clinical phenotype of these tumours.

3. Methods

Gene expression analysis on normal pituitary, AIP mutation positive, familial *AIP*neg as well as sporadic somatotrophinomas (n=25) using the Affymetrix human Gene Chip HG-U133 Plus 2.0 array. Ingenuity Pathway Analysis (IPA) tool was used for pathway analysis. Differential expression of selected genes was validated by RT-qPCR and immunohistochemistry. *In vitro* stimulation of epithelial-to-mesenchymal transition (EMT) was performed on stable AIP-knockdown cells using forskolin and assessed the EMT markers by Western blotting. *In vitro* invasion assay was performed on AIP siRNA-knocked down BxPC3 cells using BioCoat-Matrigel invasion chambers.

4. Ingenuity Pathway Analysis

Epithelial to Mesenchymal Transition (EMT) pathway in *AIP*pos somatotroph tumours



EMT- related genes in AIPpos somatotroph tumours

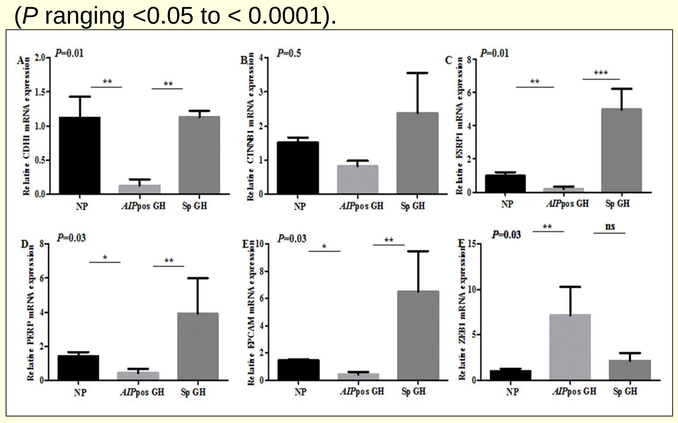
47 known EMT related genes in *AIP* pos somatotroph adenomas

Symbol	Entrez Gene Name	Affymetrix	Log fold
ADAM17	ADAM metallopeptidase domain 17	205746_s_at	1.215
AKT3	v-akt murine thymoma viral oncogene hom	242876_at	-1.545
APC	adenomatous polyposis coli	203527_s_at	1.487
BRAF	B-Raf proto-oncogene, serine/threonine ki	206044_s_at	-1.962
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	201131_s_at	-4.755
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	203440_at	-4.149
CLDN3	claudin 3	203954_x_at	-1.651
CTNNB1	catenin (cadherin-associated protein), beta	223679_at	-2.03
EGFR	epidermal growth factor receptor	224999_at	-2.499
EPCAM	epithelial cell adhesion molecule	201839_s_at	-1.28
ESRP1	epithelial splicing regulatory protein 1	225846_at	-5.01
ESRP2	epithelial splicing regulatory protein 2	219395_at	-2.565
FGF13	fibroblast growth factor 13	205110_s_at	-2.988
FGFR1	fibroblast growth factor receptor 1	222164_at	-2.212
FGFR2	fibroblast growth factor receptor 2	203638_s_at	-2.215
FGFR3	fibroblast growth factor receptor 3	204379_s_at	-3.256
FZD3	frizzled class receptor 3	239082_at	-2.878
FZD5	frizzled class receptor 5	221245_s_at	-1.89
FZD7	frizzled class receptor 7	203706_s_at	-3.946
GSK3B	glycogen synthase kinase 3 beta	226183_at	1.529
HGF	hepatocyte growth factor (hepapoietin A; s	209960_at	-2.072
HRAS	Harvey rat sarcoma viral oncogene homolo	212983_at	-1.074
JAG2	jagged 2	32137_at	-1.572
JAK1	Janus kinase 1	239695_at	-2.142
LEF1	lymphoid enhancer-binding factor 1	221558_s_at	1.836
LOX	lysyl oxidase	215446_s_at	1.464
MAP2K5	mitogen-activated protein kinase kinase 5	209756_at	1.008
MMP2	matrix metallopeptidase 2 (gelatinase A, 7	201069_at	2.307
MMP9	matrix metallopeptidase 9 (gelatinase B, 92	203936_s_at	1.495
NOTCH2	notch 2	202443_x_at	-2.306
PERP	PERP, TP53 apoptosis effector	222392_x_at	1.90
PIK3C2A	phosphatidylinositol-4-phosphate 3-kinase	241905_at	-2.88
PIK3C3	phosphatidylinositol 3-kinase, catalytic sub	232086_at	1.943
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-k	217620_s_at	-1.268
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-k	239294_at	2.083
PSENEN	presenilin enhancer gamma secretase subu	218302_at	1.548
RELA	v-rel avian reticuloendotheliosis viral onco	201783_s_at	-1.072
RRAS2	related RAS viral (r-ras) oncogene homolog	212589_at	-2.044
SMAD2	SMAD family member 2	203076_s_at	1.324
SMAD3	SMAD family member 3	218284_at	-2.046
TCF4	transcription factor 4	212385_at	1.548
TCF7L1	transcription factor 7-like 1 (T-cell specific,	221016_s_at	-1.271
TGFB2	transforming growth factor, beta 2	209909_s_at	3.127
TWIST1	twist family bHLH transcription factor 1	213943_at	-2.274
WNT4	wingless-type MMTV integration site famil	208606_s_at	2.816
WNT5A	wingless-type MMTV integration site famil	213425_at	-2.233
ZEB1	zinc finger E-box binding homeobox 1	210875_s_at	1.864

Validated genes are highlighted in the table:
Green = down
Red = up

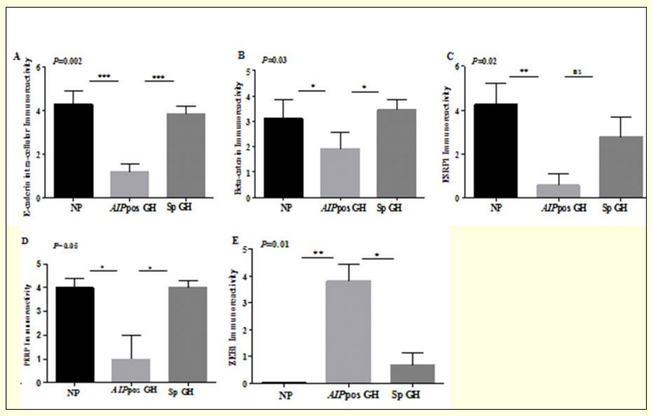
6. Validation by RT-qPCR

Validation of five downregulated (*CDH1, CTNNB, ESRP1, PERP* and *EPCAM*) and one upregulated (*ZEB1*) genes (*P* ranging <0.05 to < 0.0001).



7. Validation by IHC

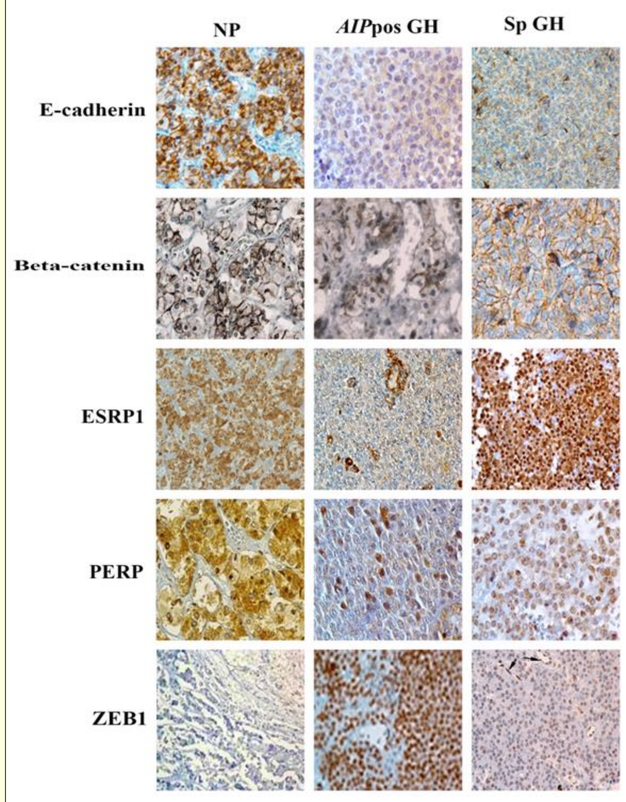
Validation at protein level for four downregulated (E-cadherin, Beta-catenin, ESRP1 and PERP) and one upregulated (ZEB1) genes (*P* ranging <0.05 to < 0.0001).



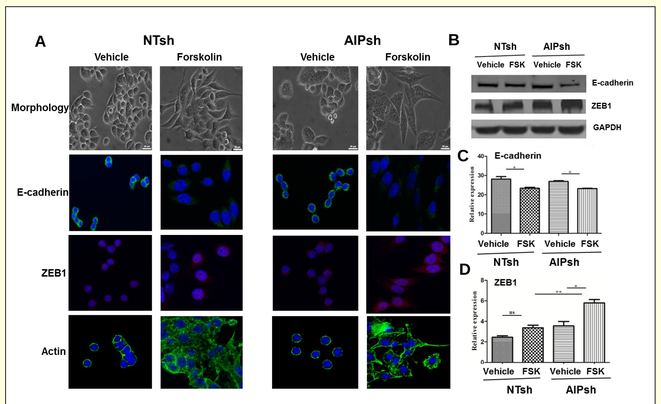
7. Validation by IHC (cont.)

Representative images:

Normal pituitary (left panel), AIPpos GH (middle panel) and sporadic GH (right panel)



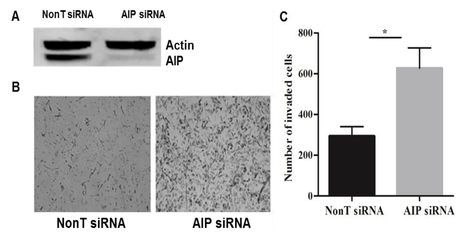
8. *In vitro* stimulation of EMT



Morphologic and phenotypic EMT-like changes in response to forskolin treatment.

A. Morphologic changes in control NT shRNA and AIP shRNA transduced GH3 cells (phase, top row) and immunofluorescence of E-cadherin (green, second row), ZEB1 (red, third row) and actin (green, bottom row) at 72h, after treating the cells with vehicle or FSK (10μM for 30 min). **B**. Shows differential expression of EMT markers by Western blotting. **C** and **D**. Densitometric analysis of E-cadherin and ZEB1 expression. *P* values indicated < 0.05 (*) and < 0.01 (**); one-way ANOVA for multiple comparisons.

9. Invasion assay



A. Western blot showing knockdown of AIP in BxPC3 cells. Actin was used as a loading control. **B**. Photographs showing cells treated with non-targeting siRNA or AIP siRNA invading through 8-micron pores in a Matrigel invasion chamber after 48h. **C**. Mean (± SEM) number of invading cells/chamber (n=9). More invading cells are seen after AIP silencing than non-targeting (NonT) control siRNA (*P*<0.05).

10. Summary and Conclusions

One of the top altered pathways in *AIP*pos adenomas was the EMT pathway. Genes related to EMT, such as epithelial markers (CDH1, CTNNB1, ERSP1 and EPCAM), transcriptional regulator (ZEB1) and post-transcriptional regulator (ESRP1 and ESRP2) all appear to be significantly deregulated.

The cAMP pathway has tissue specific regulation on cell proliferation[3] and possibly on EMT. We hypothesise that increased levels of cAMP could stimulate EMT in the pituitary, while it inhibits in other cell types [4].

In vitro EMT stimulation lead to induction of EMT as indicated by down-regulation of epithelial marker and up-regulation of mesenchymal marker (ZEB1) as well as an increase in actin stress fibers formation. Invasion assay revealed that AIP silencing led to an increase in invasion compared to non-targeting siRNA.

This novel potential mechanism of the regulation of EMT/or switching the cellular phenotype from 'epithelial' to 'mesenchymal like' through AIP may thus be important for acquiring an invasive phenotype.

11. References

1 Daly AF, *et al* (2010) *J.Clin.Endocrinol.Metab.*, **95**, E373-E383.
2. Chahal, H, *et al* (2012) *J.Clin.Endocrinol.Metab.*, **97**, E1411-1420.
3. Stork PJ, *et al* **(**2002) *Trends in Cell Biology* **12**:258-266
4. Zhang A et al, (2006) *American Journal of Physiology. Renal Physiology* **291**:F1323-1331

12. Acknowledgement

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