



Whole exome sequencing of adenoid cystic carcinoma

Philip J. Stephens,¹ Helen R. Davies,¹ Yoshitsugu Mitani,² Peter Van Loo,^{1,3} Adam Shlien,¹ Patrick S. Tarpey,¹ Elli Papaemmanuil,¹ Angela Cheverton,¹ Graham R. Bignell,¹ Adam P. Butler,¹ John Gamble,¹ Stephen Gamble,¹ Claire Hardy,¹ Jonathan Hinton,¹ Mingming Jia,¹ Alagu Jayakumar,¹ David Jones,¹ Calli Latimer,¹ Stuart McLaren,¹ David J. McBride,¹ Andrew Menzies,¹ Laura Mudie,¹ Mark Maddison,¹ Keiran Raine,¹ Serena Nik-Zainal,¹ Sarah O'Meara,¹ Jon W. Teague,¹ Ignacio Varela,¹ David C. Wedge,¹ Ian Whitmore,¹ Scott M. Lippman,⁴ Ultan McDermott,¹ Michael R. Stratton,¹ Peter J. Campbell,¹ Adel K. El-Naggar,² and P. Andrew Futreal¹

¹Cancer Genome Project, Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom.

²Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. ³Human Genome Laboratory, Department of Human Genetics, VIB and KU Leuven, Leuven, Belgium. ⁴Department of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

Adenoid cystic carcinoma (ACC) is a rare malignancy that can occur in multiple organ sites and is primarily found in the salivary gland. While the identification of recurrent fusions of the *MYB-NFIB* genes have begun to shed light on the molecular underpinnings, little else is known about the molecular genetics of this frequently fatal cancer. We have undertaken exome sequencing in a series of 24 ACC to further delineate the genetics of the disease. We identified multiple mutated genes that, combined, implicate chromatin deregulation in half of cases. Further, mutations were identified in known cancer genes, including *PIK3CA*, *ATM*, *CDKN2A*, *SF3B1*, *SUFU*, *TSC1*, and *CYLD*. Mutations in *NOTCH1/2* were identified in 3 cases, and we identify the negative *NOTCH* signaling regulator, *SPEN*, as a new cancer gene in ACC with mutations in 5 cases. Finally, the identification of 3 likely activating mutations in the tyrosine kinase receptor *FGFR2*, analogous to those reported in ovarian and endometrial carcinoma, point to potential therapeutic avenues for a subset of cases.

Introduction

Adenoid cystic carcinoma (ACC) is a rare salivary gland malignancy characterized by an indolent clinical course with multiple local recurrences. Even with effective local control, the majority of patients succumb to metastatic disease within a decade. Thus, there is a pressing need for new therapies fueled by improved insights into the biology of the disease. Here we report the exome sequencing analyses of 24 cases of ACC and the elaboration of the genetics of this rare cancer.

Only a handful of point mutations in known cancer genes have been reported in ACC, including *KRAS*, *BRAF*, and *TP53* (1). Recent progress has come in the identification of a recurrent translocation t(6;9)(q22-23;p23-24) resulting in the fusion of the v-myb myeloblastosis viral oncogene homolog (*MYB*) gene on chromosome 6 to the nuclear factor I/B (*NFIB*) gene on chromosome 9 in the majority of cases (2). However, the extent to which other genes are contributing to the disease and thus constitute additional targets for potential therapeutic exploitation is not well characterized.

Results and Discussion

Twenty-three pretreatment primary ACC specimens, 1 local-regional lymph node metastasis (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI67201DS1), and corresponding matching normal salivary gland parenchymal samples were subjected to solution phase

capture and next-generation sequencing of the coding exome as well as evaluation for and validation of somatic mutations as previously described (3). The case series consisted of 19 that were scored as positive for MYB activation and comprised a roughly equal mixture of cribriform and predominantly solid histological forms of ACC (Supplemental Table 1). The latter are associated with increased risk of local-regional relapse, distant metastases, and poorer overall survival (4).

Exome sequencing identified 312 somatic mutations, ranging from 2 in PD3198a to 35 in PD3181a (Supplemental Table 2), with a mean of 13 mutations per exome, lower than that reported for most adult solid tumors thus far. The mutations comprised 182 missense, 59 silent, 14 nonsense, 45 frameshift, 6 in-frame insertion/deletion, and 6 essential splice site. There was no statistically significant difference between the average numbers of somatic mutations in different histological subtypes, nor between MYB-positive and -negative cases. Recurrent losses of 1p36, 6q, 9p, 12q were noted from SNP arrays (Supplemental Table 3 and Supplemental Figure 1), in agreement with previous work (5, 6). A substantial proportion of cases with likely MYB activation also demonstrated breakpoints and copy number changes on SNP6 arrays at *MYB* and *NFIB* loci (Supplemental Table 3), potentially accounting for a substantial proportion of the reported involvement of these regions.

Somatic mutations were identified in multiple known cancer genes (Figure 1) including a *CDKN2A* truncating frameshift mutation. Further analyses of SNP6-derived copy number data identified 3 additional cases (Supplemental Table 4) with loss of heterozygosity encompassing the *CDKN2A* locus where there was no apparent involvement of the nearby *NFIB* locus in a *MYB*

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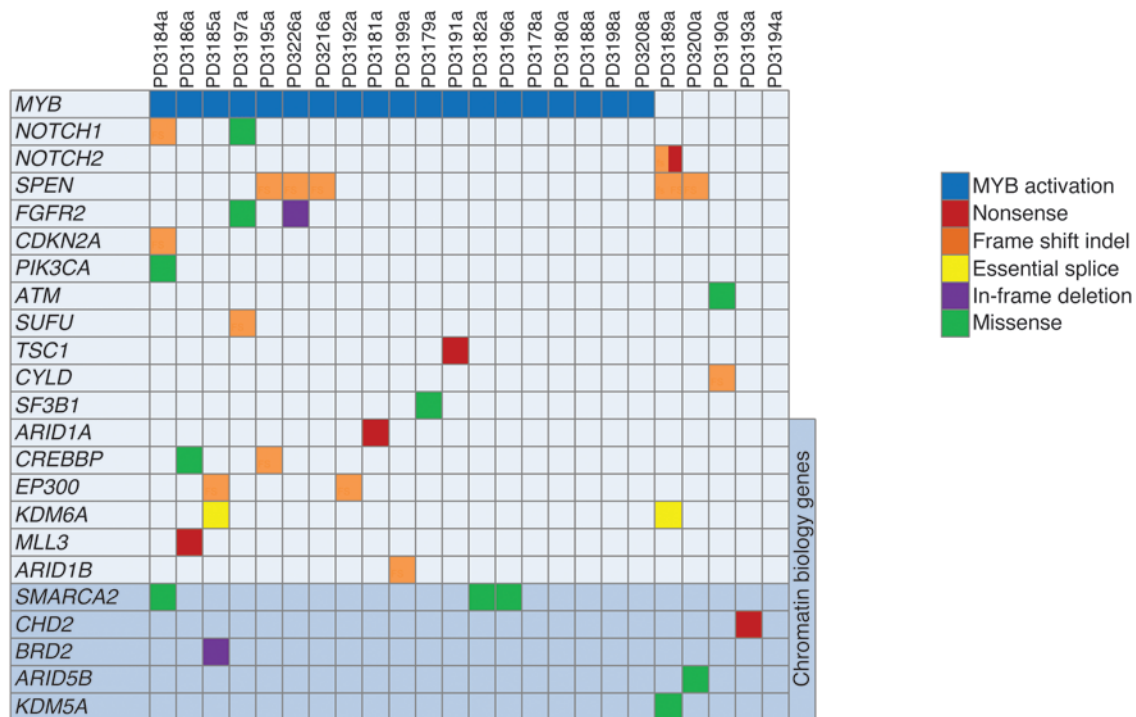
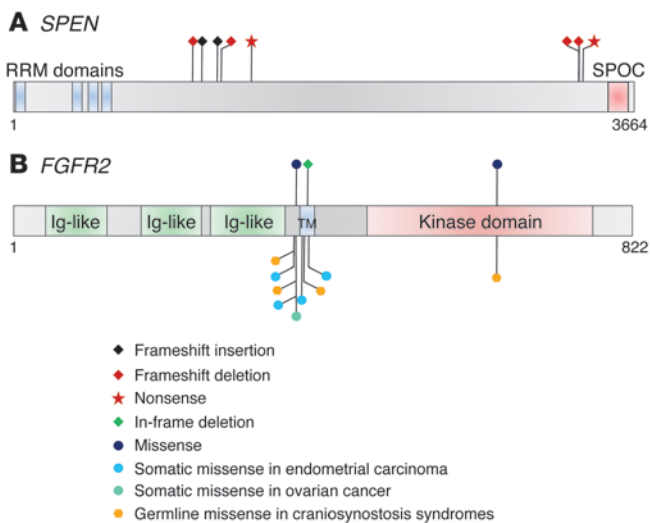


Figure 1 Somatic mutations identified in known cancer genes, *SPEN*, and genes involved in chromatin biology. The genes are listed on the left hand side and the adenoid cystic samples across the top. The darker shading at the bottom of the figure indicates genes involved in chromatin biology that have not previously been implicated in cancer.

fusion event. These data would be consistent with *CDKN2A* playing a role in a proportion of ACC. We also identified a canonical activating mutation in *PIK3CA* (p.H1047L) and a missense mutation in the *ATM* kinase (p.R337C). Arginine 337 is highly conserved and amino acid substitution mutations, p.R337S/H/C, have been reported in colorectal cancer and B cell chronic lymphocytic leukemia (1). The pattern of recurrence and multiple substitution is consistent with this mutation being likely oncogenic. Somatic truncating mutations were identified in *SUFU*, *TSC1*, and *CYLD*, 3 cancer genes associated with germline susceptibility to rare tumor syndromes as well as in the recently reported splicing factor cancer gene *SF3B1* (7).

Somatic mutations were also identified in *NOTCH1* and *NOTCH2* in 3 cases. *NOTCH1* had a missense (p.F1702S) and frameshift mutation (p.Y550fs*81) in 2 cases, while *NOTCH2* had 2 truncating mutations (p.Q2308fs*5, p.E2420*) in a single case. Phe1702 of *NOTCH1* falls within a region frequently targeted in T-ALL for activating mutations, while the truncating mutation is upstream of reported activating truncations. Recent identification of frequent truncating mutations in head and neck cancers (head and neck squamous cell carcinoma [HNSCC]) in this same region suggested that *NOTCH1* can act as a tumor suppressor gene (8, 9). The presumptive compound heterozygous truncating *NOTCH2* mutations in PD3189 are reminiscent of those reported in Hajdu-Cheney syndrome (MIM 102500) (10), which are suggested to be activating. Thus, the functional nature of *NOTCH* gene mutations in ACC is not easily discerned from the mutational pattern and may be different depending on the context of other cancer mutations in play.

Evidence was also found for alterations of genes involved in histone modification and chromatin remodeling previously implicated in cancer. Somatic mutations have been reported in *ARID1A* in clear cell ovarian, renal (ccRCC), transitional cell bladder (TCC) and gastric carcinoma (3, 11–13), *CREBBP* and *EP300* in non-Hodgkin lymphoma (NHL) (14), and *KDM6A* (*UTX*) in ccRCC and TCC (3, 11). Each of these genes had only truncating mutations identified, with the exception of a truncating and missense mutation in *CREBBP*. The missense mutation, p.R1446C, occurs in the KAT11 domain and Arg1446 is exquisitely conserved through to plants, supporting its functional importance. Three previous substitution mutations at this residue have been reported (1). Like *ATM*, the recurrent targeting of the same amino acid for multiple substitutions suggests an oncogenic phenotype for these mutations. Truncating mutations were also identified in *MLL3*, an H3K4 methylase with mutations previously reported in medulloblastoma and TCC (11, 15), and in *ARID1B*, a component of the switch/sucrose non-fermentable chromatin remodeling complex (SWI/SNF) complex like *ARID1A* with recurrent mutations recently identified in breast cancer (16). *SMARCA2*, which encodes the BRM ATPase subunit of the SWI/SNF complex was also implicated by 3 somatic missense mutations. Also likely affecting chromatin regulation were a truncating mutation in *CHD2* (a chromodomain helicase ATPase), an in-frame deletion in *BRD2* (a bromodomain protein which binds acetylated lysine residues of histone H4), missense mutations in *ARID5B* (an ARID domain family member implicated in childhood acute lymphoblastic leukemia; ref. 17) and *KDM5A*, an H3K4 demethylase and com-

**Figure 2**

SPEN and *FGFR2* are cancer genes in ACC. Representation of the somatic mutations identified in *SPEN* and *FGFR2* relative to the protein coding sequence and major domains (A) indicating the position of somatic mutation identified in this study in *SPEN*. (B) Three somatic mutations identified in *FGFR2* in this study are shown above the schematic; below are shown somatic mutations previously reported in endometrial and ovarian cancer and germline mutations in craniosynostosis syndromes.

ponent of the polycomb repressor complex 2. Summing over the entire exome series, 12/24 cases have mutations in genes directly involved in chromatin biology.

One important aspect of genomic studies of rare tumor types is the potential identification of novel cancer genes that may be prevalent only in those cancers, shedding light on facets of cancer biology that might otherwise remain obscure. In this regard, Spen homolog transcriptional regulator (*SPEN*), an inducible transcriptional regulator on chromosome 1p36, was identified as a cancer gene in ACC with 6 truncating mutations in 5 cases (all of solid histology) in the exome screen. *SPEN* encodes a transcriptional repressor with RNA-binding domains and is a regulator of NOTCH signaling, forming a complex with RBP-J to downregulate target genes in the absence of activated NOTCH signaling (18). In addition, *SPEN* interacts with SRA, an RNA nuclear receptor coactivator, to repress nuclear hormone receptor activity in the absence of ligand (19). All of the mutations would truncate the putative protein prior to the SPOC domain (Figure 2A), essential for transcriptional repression and likely for homodimerization (20). Three of 5 cases had evidence for loss of heterozygosity (LOH). One case (PD3195) was found to have 2 truncating mutations: presumptively a compound heterozygote or 2 subclones consistent with there being no discernible LOH. A fifth case, PD3189, had no LOH at the level of SNP6.0 resolution and was likely heterozygous. Thus, a simple loss-of-function model is unlikely to be operative. Intriguingly, this case also had 2 *NOTCH2* mutations as indicated above, suggesting a strong drive to abrogate NOTCH signaling. No clear correlation of *SPEN* transcript expression and mutation status was obtained from quantitative RT-PCR (qRT-PCR) data (Supplemental Figure 2) or array-based data (data not shown) across all samples — with all mutant cases demonstrating continued expression. Sequencing

of *SPEN* through a further 42 cases identified 2 additional *SPEN* truncating mutations (p.R1403*, p.Q3355*), both in solid histology cases, thus confirming the importance of *SPEN* mutations in a proportion of ACC in general and in the poor prognosis solid histology subtype in particular. We were not able to assess LOH for these 2 cases. Recent work has identified truncating *SPEN* and *NOTCH2* mutations in splenic marginal zone lymphoma (21). Of note, none of the *SPEN* mutations reported had evidence for loss of the wild-type allele, similarly arguing against a simple loss-of-function mechanism for *SPEN* mutation in this cancer type. Given the identification of *SPEN* and *NOTCH* mutations in ACC, it is reasonable to suggest that deregulation of NOTCH signaling is playing a role in a proportion of cases. These data suggest an opportunity to explore NOTCH pathway targeting as a potential therapeutic angle in ACC.

Of particular interest from a potential therapeutic aspect was the identification of mutations in *FGFR2*, encoding a receptor tyrosine kinase for basic/acidic and keratinocyte growth factors. Two mutations, p.Y376C and p.I389_V393>M, were identified in PD3197a and PD3226a. The p.Y376C mutation is identical to that observed in Beare-Stevenson cutis gyrata syndrome (MIM 123790), one of the autosomal dominant craniosynostotic syndromes characterized by kinase-activating mutations in *FGFR* family members. Mutations at the paralogous residue in *FGFR3*, p.Y373C, give rise to thanatophoric dysplasia type I (MIM 187600). Somatic p.Y376C mutations have been reported in endometrial and ovarian carcinoma (ref. 1 and Figure 2B). This mutation and analogous substitutions have been shown to be activating through constitutive receptor dimerization via creating unpaired cysteine residues resulting in ligand-independent receptor activation (22, 23). The in-frame deletion occurs in the transmembrane domain and is likely similar by analogy with other activating mutations (22). Sequencing of *FGFR2* through a further 25 cases identified a p.K642R mutation in the kinase domain identical to that described in Pfeiffer syndrome (MIM 101600), confirming the importance of *FGFR2* activating mutations in a subset of ACC. There are currently several small molecule inhibitors of FGFRs in clinical trial. Indeed, a trial of one of these, Dovitinib, in ACC has recently opened (24). It would be of considerable interest to evaluate *FGFR2* mutation status in tumors of participants.

In summary, systematic analyses of ACC genomics have uncovered the involvement of multiple cancer genes that are likely to be contributing to tumor development in the context of MYB activation. These data point to disruption of chromatin regulation as a major factor in ACC development. Further, identification of *SPEN* and *NOTCH* mutations implicate NOTCH signaling deregulation in a proportion of cases. Finally, these analyses have identified activating mutations of the receptor tyrosine kinase *FGFR2* in a proportion of cases, thus suggesting a potential therapeutic point of attack on this rare but lethal cancer.

Methods

Diagnosis of ACC. The diagnosis of ACC was only made based on the presence in any part of the tumor of a tubular and/or cribriform patterns, regardless of the presence and extent of the solid component.

Assessment of MYB fusion status. *MYB-NFIB* fusion status was investigated using a combination of fusion transcript sequencing, qRT-PCR, FISH, and 3' rapid amplification of cDNA ends (3' RACE), as described previously (25). The particular data supporting a scoring of MYB activation in any given sample are given in Supplemental Table 1.



brief report

Exome sequencing and variant detection. See Supplemental Methods for details of exome enrichment, sequencing, variant detection, and validation. The sequence data have been deposited at the European Genome-phenome Archive (EGA00001064049).

Detection of copy number variation. SNP array hybridization on the Affymetrix SNP6.0 platform was as per Affymetrix Protocols. Copy number analysis was performed using ASCAT (version 2.1) accounting for nonneoplastic cell admixture and tumor aneuploidy (26) and resulted in integer allele-specific copy number profiles for the tumor cells. The data have been deposited in Array Express (E-MTAB-1141).

Expression array analysis. See Supplemental Methods for details of the expression array analysis and *SPEN* expression by qRT-PCR. The expression array data have been deposited in Array Express (E-MTAB-1397).

Statistical assessment of the effects of histology and MYB status. A generalized linear model with negative binomial distributions was used to fit the number of mutations using the predictor variables (a) histological subtype and (b) MYB status. Neither variable was a significant predictor of the number of mutations (*P* values = 0.34 and 0.28, respectively). *P* < 0.05 was considered significant.

Study approval. Collection and use of patient samples for this study was approved by the appropriate local IRB and further local research ethics committee (LREC) approval was obtained for this genomic study specifically. Patient samples were obtained under a waiver of informed consent from the IRB.

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Address correspondence to: Adel K. El-Naggar, The Department of Pathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 085, Houston, Texas 77030, USA. Phone: 713.792.3109; Fax: 713.745.1105; E-mail: anaggar@mdanderson.org. Or to: Andrew Futreal, Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Unit 1954, P.O. Box 301429, Houston, Texas 77230, USA. Phone: 713.794.4764; Fax: 713.792.9636; E-mail: afutreal@mdanderson.org.

Andrew Futreal's present address is: Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

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