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Mutation profiling of adenoid cystic carcinomas from multiple anatomical sites identifies mutations in the RAS pathway, but no *KIT* mutations

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Abstract

Aims—The majority of adenoid cystic carcinomas (AdCCs), regardless of anatomical site, harbour the *MYB-NFIB* fusion gene. The aim of this study was to characterize the repertoire of somatic genetic events affecting known cancer genes in AdCCs.

Methods and results—DNA was extracted from 13 microdissected breast AdCCs, and subjected to a mutation survey using the Sequenom OncoCarta Panel v1.0. Genes found to be mutated in any of the breast AdCCs and genes related to the same canonical molecular pathways, as well as *KIT*, a proto-oncogene whose protein product is expressed in AdCCs, were sequenced in an additional 68 AdCCs from various anatomical sites by Sanger sequencing. Using the

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Conflict of interest

The authors declare no conflict of interest.

Sequenom MassARRAY platform and Sanger sequencing, mutations in *BRAF* and *HRAS* were identified in three and one cases, respectively (breast, and head and neck). *KIT*, which has previously been reported to be mutated in AdCCs, was also investigated, but no mutations were identified.

Conclusions—Our results demonstrate that mutations in genes pertaining to the canonical RAS pathway are found in a minority of AdCCs, and that activating *KIT* mutations are either absent or remarkably rare in these cancers, and unlikely to constitute a driver and therapeutic target for patients with AdCC.

Keywords

adenoid cystic carcinoma; *BRAF*; *HRAS*; *KIT*; sequencing

Introduction

Adenoid cystic carcinomas (AdCCs) are malignant tumours that often affect the salivary glands, but can also occur in other anatomical sites, such as the lung and breast.¹ Salivary gland AdCC is the most prevalent type of salivary gland carcinoma,² and presents as a slow-growing but aggressive cancer, frequently resulting in multiple local recurrences and the development of distant metastases. Patients with salivary gland AdCC have a poor long-term outcome.³ In contrast, breast AdCCs show an indolent clinical behaviour, with a 90–100% 10-year survival rate and a low incidence of distant metastases.^{1,4,5}

AdCCs provide a clear example of genotypic–phenotypic correlation, as they have similar morphological characteristics and genetic aberrations, independently of the site of origin. Recent studies have demonstrated that a subgroup of AdCCs are characterized by the presence of a recurrent t(6:9)(q22–23;p23–24) chromosomal translocation.⁶ This translocation generates a fusion transcript involving the oncogene *MYB* and the transcription factor gene *NFIB*. In the resulting fusion gene, the *MYB* 3′-untranslated region (UTR) is replaced by the *NFIB* 3′-UTR, leading to loss of microRNA-mediated down-regulation of *MYB*.⁶ The prevalence of the fusion gene is reported to range from 30–100% in salivary gland AdCCs^{6–9} to 90–100% in breast AdCCs.^{6,10} Even in AdCCs lacking the *MYB*–*NFIB* fusion gene, *MYB* has been shown to be consistently overexpressed in AdCCs,¹⁰ indicating that other mechanisms resulting in *MYB* overexpression are operational in these tumours. These observations provide circumstantial evidence that *MYB* is likely to constitute a driver of AdCCs, regardless of the anatomical site.

The genetic features of salivary gland and breast AdCCs have been investigated, and these tumours have been shown to harbour low levels of genetic instability, frequent loss of 6q and 9p, and a low frequency of high-level amplification of cancer-related genes.^{10–13} In salivary glands, amplification of *CCND1*, *MDM2* and *CTTN* has been reported, but only in up to 5% of cases.^{11,12} No recurrent amplifications have been reported in breast AdCCs.^{10,14} On the basis of these observations, it is plausible that mechanisms other than gene copy number aberrations drive the diverse clinical behaviour of AdCCs. In fact, other oncogenic events have been described in AdCCs,¹⁵ including mutations of *KIT*,^{15,16} *PIK3CA*, and *PTEN*.¹⁷ Currently, there is no additional information on the prevalence of

mutations affecting the components of the phosphoinositide-3-kinase (PI3K) canonical pathway in AdCCs or on the mutational repertoire of breast AdCCs.

The tyrosine kinase receptor c-KIT is expressed in up to 100% of AdCCs of the breast^{15,18} and salivary glands,¹⁹ and has been proposed as a driver and potential therapeutic target in AdCCs.¹⁵ The efficacy of therapies targeting c-KIT, in particular of imatinib mesylate, appears to be restricted to patients whose tumours harbour a mutant *KIT* gene.²⁰ The prevalence of *KIT* mutations in AdCC has been investigated in several studies,^{15,16,19,21–24} and has proven to be controversial. Although independent investigators have failed to identify *KIT* mutations in AdCCs,^{19,21–24} two studies reported *KIT* mutations in 12% and 88% of formalin-fixed paraffin-embedded (FFPE) salivary gland AdCCs.^{15,16} Although case reports of AdCCs have suggested a benefit from imatinib,^{25,26} clinical trials assessing imatinib as targeted therapy in patients with AdCC have yielded negative results.^{22,27} Thus, currently, no specific targeted therapy exists for the treatment of patients with breast or salivary gland AdCCs.

The aims of this study were to investigate whether breast AdCCs harbour point mutations in 19 known cancer-related genes,^{28–32} and to investigate the prevalence of *KIT* mutations in a series of breast, lung and salivary gland AdCCs.

Materials and methods

Case Selection

A series of 26 fresh-frozen and 65 FFPE primary and metastatic AdCCs of the breast ($n = 25$), lung ($n = 7$), head and neck ($n = 56$), pancreas ($n = 1$), kidney ($n = 1$) and vaginal wall ($n = 1$) were retrieved from the tissue banks of the Royal Marsden Hospital, London, UK, the Cleveland Clinic, Ohio, USA, the Curie Institute, Paris, France, the Royal Surrey County Hospital, Guildford, UK, and the Department of Pathology, Sahlgrenska Cancer Centre, Gothenburg, Sweden (Tables 1 and S1). Cases were anonymized prior to inclusion in this study, and approval by the local ethics committees was obtained. Cases were locally reviewed by the contributing pathologists, and centrally reviewed by two of the authors (D.N.R. and J.S.R.-F.). Only bona fide AdCCs were included in this study.

Microdissection and DNA Extraction

To ensure >90% purity of neoplastic cells, microdissection was performed with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan) from four to 10 consecutive 8- μ m-thick sections stained with nuclear fast red, as previously described.³³ DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK), as previously described.³³ The double-stranded DNA concentration was measured using the Picogreen assay, according to the manufacturer's instructions (Invitrogen, Paisley, UK). Ten cases did not yield DNA of sufficient quantity and/or quality, and were excluded from further analyses.

Genotyping With Sequenom OncoCarta

Thirteen AdCCs of the breast were subjected to mutation screening using the OncoCarta Panel v1.0 (Sequenom, San Diego, CA, USA), consisting of 24 pools of polymerase chain reaction (PCR) primer pairs and extension primers with the capacity to interrogate 238 known mutations in 19 cancer-related genes (*ABL1*, *AKT1*, *AKT2*, *BRAF*, *CDK4*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *JAK2*, *KIT*, *MET*, *HRAS*, *KRAS*, *NRAS*, *PDGFA*, *PIK3CA*, and *RET*). In brief, 20 ng of DNA was amplified using the 24 sets of OncoCarta PCR primer pools. Subsequently, a single base extension reaction using the OncoCarta extension primers was performed. After using a cation exchange resin to remove salts, the products were spotted, using the nanodispensing system, on a 384 SpectroChipII, and analysed on a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Sequenom). Data were interrogated using MassARRAY TYPER analyser software 4. Mutations were manually reviewed by use of the visual and the raw spectrum patterns. The prevalence of mutant alleles was estimated by calculating the ratio of the height of the raw spectrum of the mutant allele to that of its wild-type allele.

Sanger Sequencing

Mutations with an allelic ratio $\geq 25\%$, as defined by the Sequenom MassARRAY assay, were validated in the index cases by Sanger sequencing, where primers were designed using PRIMER3 (<http://frodo.wi.mit.edu/primer3/>), as previously described.³⁴ Mutations validated in the index cases were tested in an additional 68 AdCC cases (48 head and neck, seven lung, 12 breast, and one vaginal wall) from which DNA of sufficient quantity and quality could be extracted. In addition, the presence of activating mutations affecting codons 12, 13 and 61 of *KRAS*, *HRAS*, and *NRAS*, respectively, and exons 8, 9, 11, 13 and 17 of *KIT*, were investigated in all 68 cases (primer sequences are given in Table S2). Sequences were analysed with MUTATION SURVEYOR software (Softgenetics LLC., State College, PA, 16803 USA). Sequencing was performed twice for each sample harbouring a potential mutation, to rule out the possibility of PCR fidelity artefacts, and was carried out in both directions.

Results

To identify potential secondary driver events in AdCCs, 13 previously characterized AdCCs of the breast¹⁰ were subjected to Sequenom analysis. No mutations in cancer genes previously implicated in breast cancer (Cosmic database, <http://www.sanger.ac.uk/genetics/CGP/cosmic/>), including *AKT1*, *ERBB2*, and *PIK3CA*, were detected. Interestingly, however, *BRAF*, a gene reported to be mutated in $<1\%$ of invasive breast cancers, was found to be mutated in two breast AdCCs (15%; Figure 1; Table 2). Although both mutations identified, V600E and G464E, are reported to be activating,^{35,36} the former has been shown to be a high-activity mutant, whereas the latter is an intermediate-activity mutant.^{35,36} Both mutations were successfully validated by means of Sanger sequencing. In addition, one *KIT* mutation and one *KRAS* mutation were identified by Sequenom analysis; however, these mutations could not be validated by Sanger sequencing (data not shown). This may be because of the relatively low allelic frequency of the mutations, making confident verification with Sanger sequencing challenging. Importantly, even after repeated microdissections and alternative Sanger sequencing strategies (e.g. cDNA sequencing; data

not shown), these mutations were not validated, and should therefore be interpreted as false-positive results.

The identified *BRAF* mutations suggested potential alteration of the RAS pathway in two AdCCs. As other members of the pathway also represent targets of oncogenic mutations, we sequenced the mutational hotspots of codons 12, 13 and 61 of *KRAS*, *HRAS* and *NRAS* in addition to *BRAF* hotspot mutations (i.e. exons 8, 9, 11, 13 and 17) in an independent series of 68 AdCCs from various sites. Sanger sequencing identified an *HRAS* Q61K hotspot mutation in a head and neck AdCC, and a *BRAF* D594G kinase-dead³⁷ hotspot mutation in another breast AdCC (Figure 2). All mutations were confirmed in independent PCRs and sequencing reactions. None of the additional AdCCs investigated harboured mutations in *KRAS*. The above mutations were all identified in distinct samples (i.e. no tumour sample harboured more than one mutation in the genes tested in this study). Therefore, out of the components of the RAS pathway investigated in this study, ~4% and 1% harboured validated *BRAF* and *HRAS* mutations, respectively.

Because of the controversy surrounding the existence of activating *KIT* mutations in AdCCs, and their potential clinical implications, we sequenced exons 8, 9, 11, 13 and 17 of *KIT*. None of the AdCCs analysed here harboured a mutation in *KIT*. Power calculations revealed that, if the true prevalence of *KIT* mutations was 4% (relative to an expected prevalence of 15%), the probability of a negative result (i.e. no mutations found in 81 samples) by chance would be $<10^{-4}$, which equates to a statistical power of >95%. Therefore, a negative result is unlikely to be attributable to a type II or β error.

Discussion

AdCCs from different sites show similar patterns of genomic alterations. Here, we characterized the mutation status of known cancer genes in a large series of breast AdCCs, and selected cancer genes in a large series of AdCCs from various anatomical sites. Our results demonstrate that mutations affecting the RAS pathway are found in a subset of AdCCs. In addition, our results show that *KIT* mutations are either absent or vanishingly rare in AdCCs, suggesting that they are unlikely to constitute clinically useful therapeutic targets for this disease.

Dysregulated signalling of the RAS pathway has a major role in the development of human cancers, and activating hotspot mutations have been identified in several members of the pathway.^{38,39} The hotspot mutations of *KRAS*, *HRAS* or *NRAS* occur at codon 12, 13 or 61, and cause them to become constitutively active. Mutations of *KRAS* have been previously described in AdCCs of the salivary gland, with a prevalence of 4%.⁴⁰ Our study confirms this observation, and provides the novel observation that other components of the RAS pathway are also mutated in a small subset of AdCCs. Likewise, two of the *BRAF* mutations identified in AdCCs in this study (i.e. V600E and G464E) also result in an increase in kinase activity;^{35,36} however, the *BRAF* D594G mutation found is a kinase-dead mutation^{37,41} that results in reduction/abrogation of the kinase activity of BRAF. Activating *BRAF* mutations are found in wide range of cancers, including melanomas, and ovarian, lung and colorectal cancer.³⁶ Targeted therapy against mutated BRAF V600E exists

in the form of PLX403242 (vemurafenib; Plexxikon/Roche, Berkeley, CA, USA), and high PLX4032 response rates in patients with *BRAF*V600E mutant melanoma have been observed.⁴³ Kinase-dead *BRAF* mutations, on the other hand, are rarer. The *BRAF*D594G mutation does not lead to phosphorylation of MEK, activation of CRAF, or stimulation of cell signalling.^{35,37} Importantly, however, in *in-vitro* and animal model studies,⁴⁴ kinase-dead *BRAF* mutations such as D594G may result in paradoxical and strong activation of the MEK pathway if the pathway is activated by an event upstream of BRAF.⁴⁴ In the case of AdCCs, one could hypothesize that c-KIT activation could result in strong activation of the extracellular signal-related kinase pathway in the presence of the kinase-dead *BRAF*D594G mutation. It should be noted that, in the context of kinase-dead *BRAF* mutations, BRAF-specific inhibitors are unlikely to be effective; however, on the basis of preclinical models of melanoma, it is plausible that MEK inhibitors may potentially be of clinical benefit.⁴⁴

Although we demonstrated here that *BRAF* was recurrently mutated in breast AdCCs (~4%), and that *HRAS* was mutated in another head and neck AdCC, the mutations identified in this study account for only 5% of the AdCCs assessed. Recently, potential novel driver mutations in the mitogen-activated protein kinase (MAPK) family other than *RAS* and *BRAF* have been described, including *MAP2K4* and *MAP3K1*.⁴⁵ Further studies to determine the mutation status of *MAP2K4* and *MAP3K1* in AdCCs are warranted. Functional studies are also required to determine whether mutations of genes in the RAS pathway act as oncogenic drivers in AdCCs. Critically, however, clinical trials testing MEK-specific or BRAF-specific inhibitors in patients with AdCC will require careful patient selection and the development of robust biomarkers.

Mutations in additional oncogenes have been described in AdCCs. A previous study suggested that AdCCs may harbour mutations in the components of the PI3K pathway, and a *PTEN* mutation and a *PIK3CA* mutation (in exon 1, 169A>G, I31M) were described in one case of AdCC from the breast and the renal metastasis of the same patient;¹⁷ however, no *PIK3CA* mutations were identified in any of the breast AdCCs analysed by Sequenom analysis, and the functional effects of the *PIK3CA* I31M mutation have not been reported. The presence of activating *KIT* mutations in AdCCs has proven controversial. Although multiple studies^{19,21–24} have suggested that AdCCs do not harbour activating *KIT* mutations, Vila *et al.*¹⁵ and Tetsu *et al.*¹⁶ reported mutations affecting this oncogene in 12–88% of AdCCs. Our results do not confirm the observations of Vila *et al.* and Tetsu *et al.* In fact, none of the 81 AdCCs tested harboured *KIT* mutations, suggesting that mutations affecting this proto-oncogene are either nonexistent or vanishingly rare in AdCCs, given that the likelihood of our negative findings in 81 AdCCs being attributable to a type II or β error is remarkably low ($P < 10^{-4}$). Importantly, it should be noted that, in the studies reporting on the presence of *KIT* mutations in AdCCs,^{15,16} FFPE material was analysed, and that 19 of the 21 mutations reported by Vila *et al.*¹⁵ were C>T or A>G transitions. These patterns of single-nucleotide changes have been previously reported as potential artefacts of sequencing DNA extracted from FFPE tumours.⁴⁶ Taken together, our results and those of previous studies^{19,21–24} suggest that *KIT* mutations are either non-existent or vanishingly rare in AdCCs, and that imatinib mesylate is unlikely to be a potential targeted therapy for patients with this malignancy, given that this small-molecule inhibitor has proven to be efficacious in patients whose tumours harbour activating *KIT* mutations.

This study has a number of limitations. First, as samples were accrued from a number of centres across the world, it was not possible to retrieve sufficient clinical annotations to determine whether the mutations identified were from tumours that followed an aggressive clinical course. Second, despite our efforts in retrieving AdCCs from multiple institutions, the statistical power of the study was still limited. Third, given the retrospective nature of our study and the potential biases in sample selection, it should be perceived as hypothesis-generating. Fourth, given the multi-institutional origin of samples, the retrospective nature of the study, the various therapies that the patients received, and the incomplete follow-up information available for the patients whose tumours were included, formal survival analyses could not be performed.

Taken together, our results demonstrate that a minority of AdCCs harbour activating mutations in RAS pathway genes. Further studies investigating whether these mutations act as drivers in AdCCs and could be exploited as potential therapeutic targets for patients with this disease are warranted. Finally, our results and those from previous studies that also failed to identify activating *KIT* mutations^{19,21–24} in AdCCs from various anatomical sites call into question their very existence in AdCCs, demonstrating that c-KIT small-molecule inhibitors are unlikely to constitute clinically useful targeted therapies for patients with AdCCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mutational profiling of adenoid cystic carcinoma (AdCC) of the breast using the Sequenom MassARRAY platform. Sequenom call cluster plots and mass spectrometry profiles for the detection of the (a) *BRAF*G464E (*BRAF_G1391A*) and (b) V600E (*BRAF_T1799A*) mutations are shown, together with the Sanger sequencing validation for each affected case. Call cluster plots show the low and high mass allele ratios on the *x*-axis and *y*-axis; wild-type calls are in orange (G464E) or blue (V600E), and mutant calls are in red (circled red). Mass spectrometry profiles show the allelic mass on the *x*-axis and the intensity on the *y*-axis. The corresponding sequencing chromatogram for case 7, which harbours a wild-type *BRAF* gene (i.e. G464 and V600), is shown below the mutant case for each mutation (in black). UEP, unextended primer. The red arrow marks a mutant allele.

Figure 2.

Mutational analysis of adenoid cystic carcinoma (AdCC) from various anatomical sites by Sanger sequencing. **A**, The top panel shows the Sanger sequencing chromatogram of the *HRAS* Q61K (C181A) mutation identified in case 46, a salivary gland AdCC. The bottom panel shows case 51, a *HRAS* wild-type salivary gland AdCC. **B**, The top panel shows the Sanger sequencing chromatogram of the *BRAF* D594G (A1781G) mutation identified in

case 91, a breast AdCC. The bottom panel shows case 90, a *BRAF* wild-type breast AdCC. Red arrows mark the position of the base change.

Table 1

Summary of tumour samples analysed in this study

Anatomical site	Fixation		Analysis		
	Fresh frozen	FFPE	Sequenom	Sanger	Not analysed
Breast ($n = 25$)	10	15	13 *	14	0
Head and neck ($n = 56$)	16	40	0	48	8 *
Lung ($n = 7$)	0	7	0	7	0
Pancreas	0	1	0	0	1
Kidney	0	1	0	0	1
Vaginal wall	0	1	0	1	0

FFPE, formalin-fixed paraffin-embedded.

* All FFPE samples.

Table 2

Characteristics of tumour samples in which validated mutations were identified in this study

Case	Anatomical site	Histological pattern	Gene	Mutation (amino acid)	Allelic frequency*
1	Breast	Cribriform–solid	<i>BRAF</i>	G464E	0.25
2	Breast	Cribriform–solid	<i>BRAF</i>	V600E	0.8
46	Parotid	NK	<i>HRAS</i>	Q61K	Sanger
91	Breast	NK	<i>BRAF</i>	D594G	Sanger

NK, not known; Sanger, mutations identified and validated by Sanger sequencing; hence, their allelic frequency could not be estimated.

* For mutations identified by Sequenom MassARRAY.