SUPPLEMENTARY FIGURES AND TABLES

Genetic Hallmarks Of Recurrent/Metastatic Adenoid Cystic Carcinoma

SUPPLEMENTARY FIGURES

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Figure S1. Flow diagram of study design. MDA, MD Anderson Cancer Center; MSK, Memorial Sloan Kettering Cancer Center; Hopkins, Johns Hopkins Medicine; WES, whole exome sequencing; WGS, whole genome sequencing; tNGS, targeted next generation sequencing panel; FM, Foundation Medicine

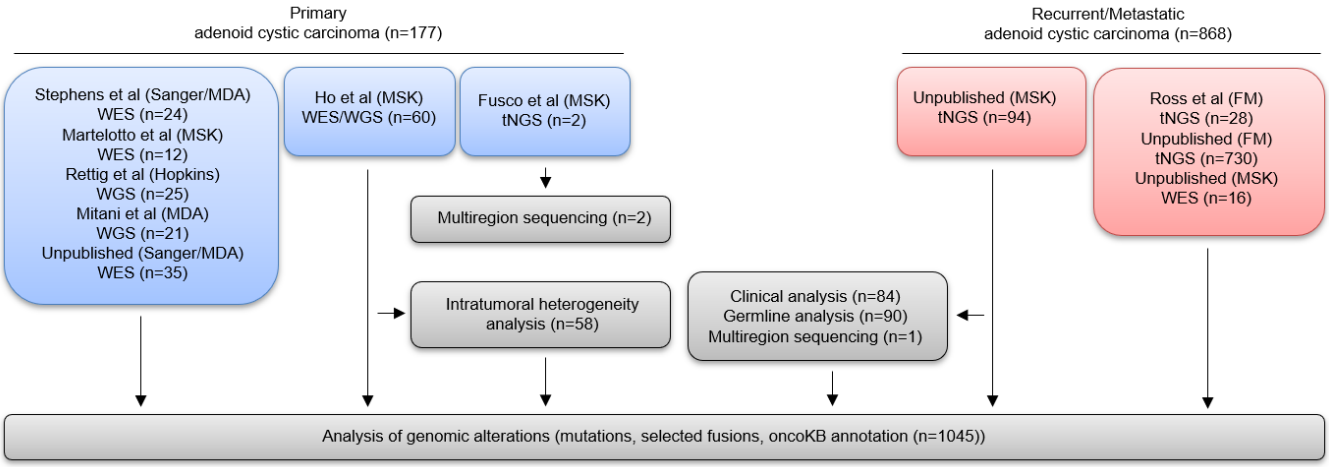

Figure S2. Primary and recurrent/metastatic adenoid cystic carcinoma distribution by anatomic site.

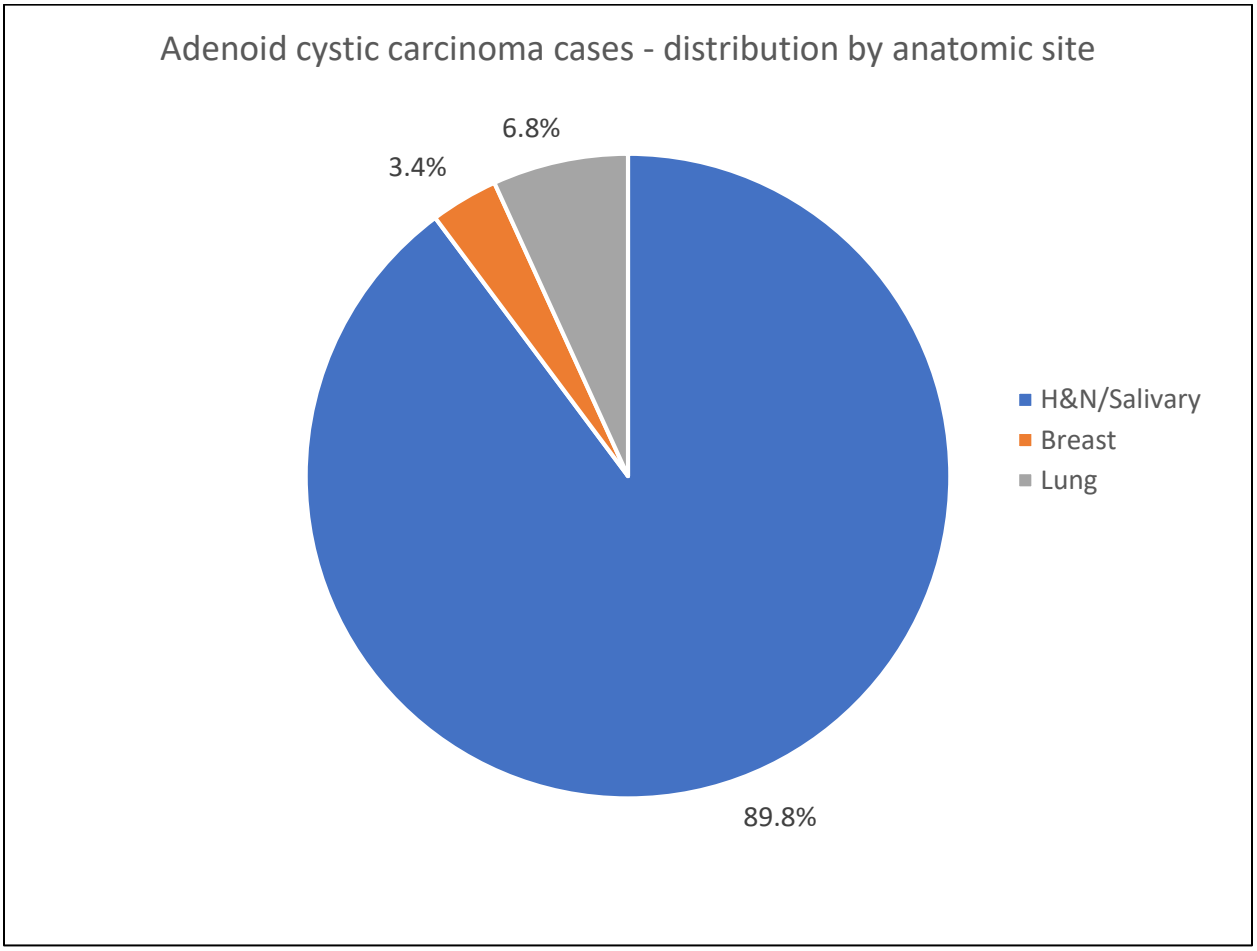

Figure S3. Variant allelic frequency (VAF) density histogram for *NOTCH1* mutations observed in recurrent/metastatic adenoid cystic carcinoma (R/M ACC). Cases with diploid *NOTCH1* copy number, demonstrating that only a small fraction of cases had potentially subclonal (VAF<0.1 in 13.5% (43/319) of cases) *NOTCH1* mutations.

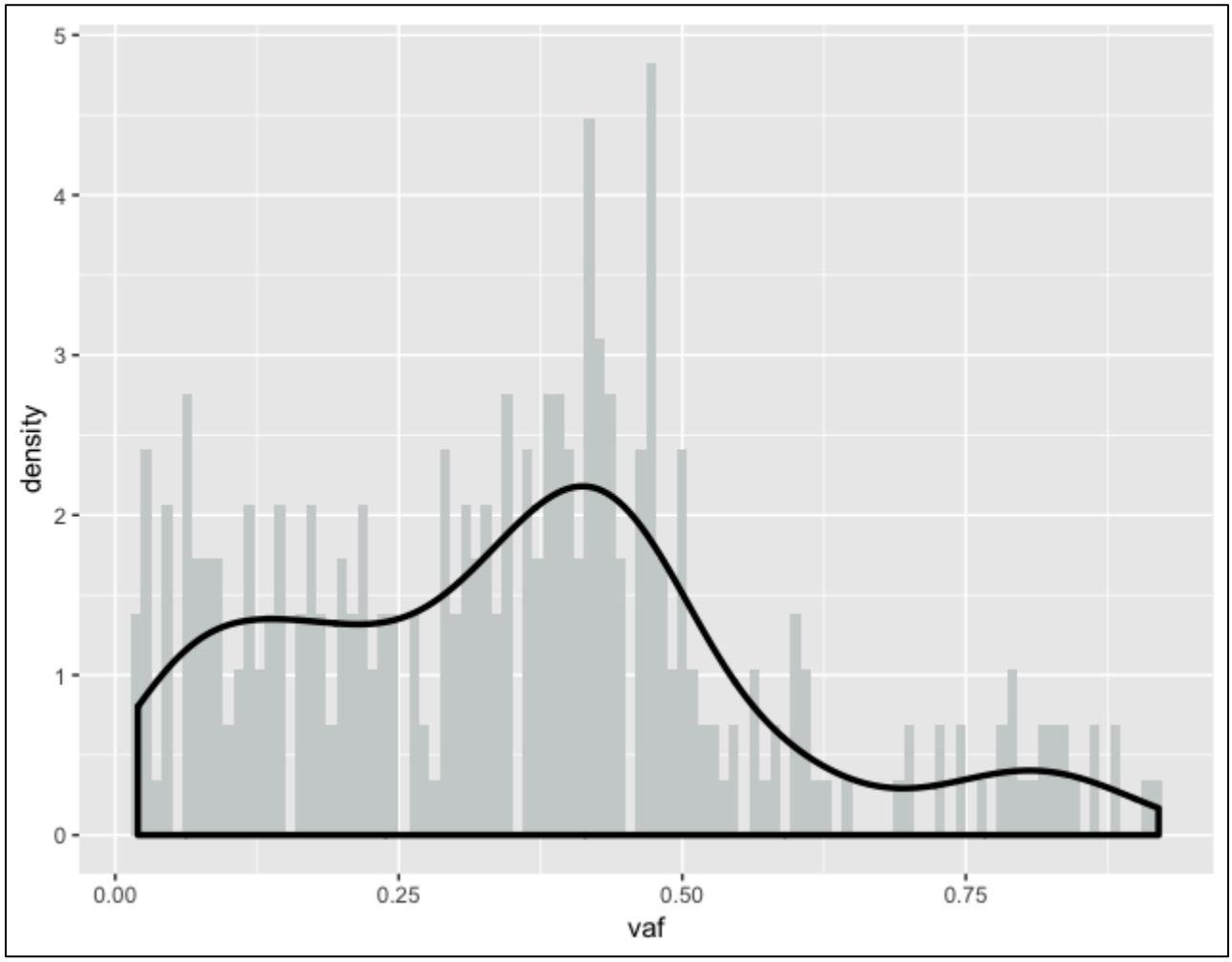

Figure S4. Downsampling analysis of R/M MSK-IMPACT cohort (n=94) to simulate mutation detection at 100x coverage. Five independent downsampled bam files from each sample were generated, which were passed through the same mutation caller and with same settings as for WES samples. This analysis showed minimal difference in VAFs between the original (~600x) and downsampled (100x) sequencing, with the VAF changed (decreased) by .011 on average in the downsampled cases. Each dot represents the average VAF, and the vertical line shows the full range (not standard deviation/error) in downsampled VAFs.

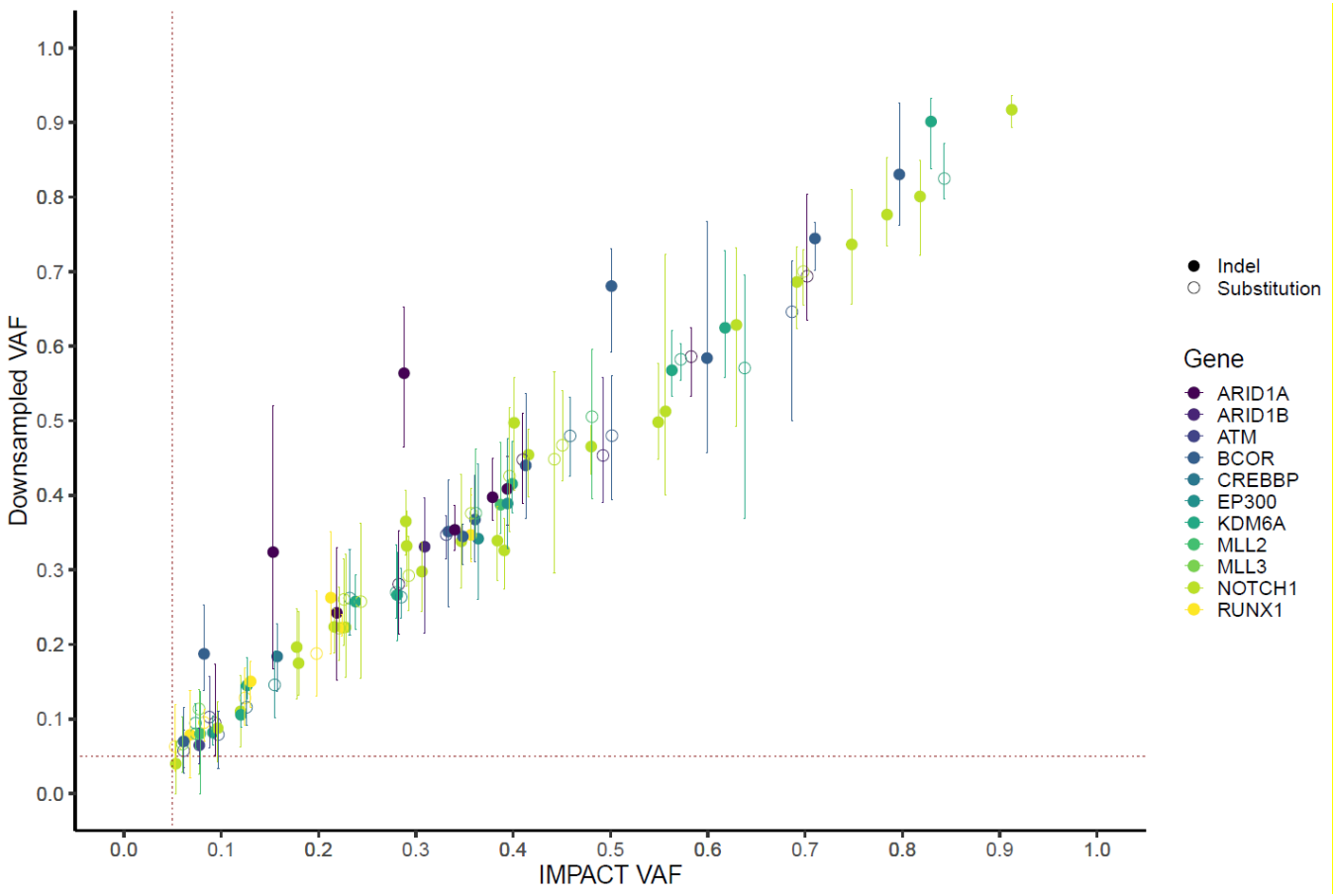

Figure S5. Representative PyClone plots demonstrating intratumoral heterogeneity quantified as number of genomically distinct subclonal populations in adenoid cystic carcinoma. (a). Sample 3492. (b). Sample 2000756. (c). Sample 148632. (d). Sample 36773720. (e). Sample D3212. (f). Sample C3070.

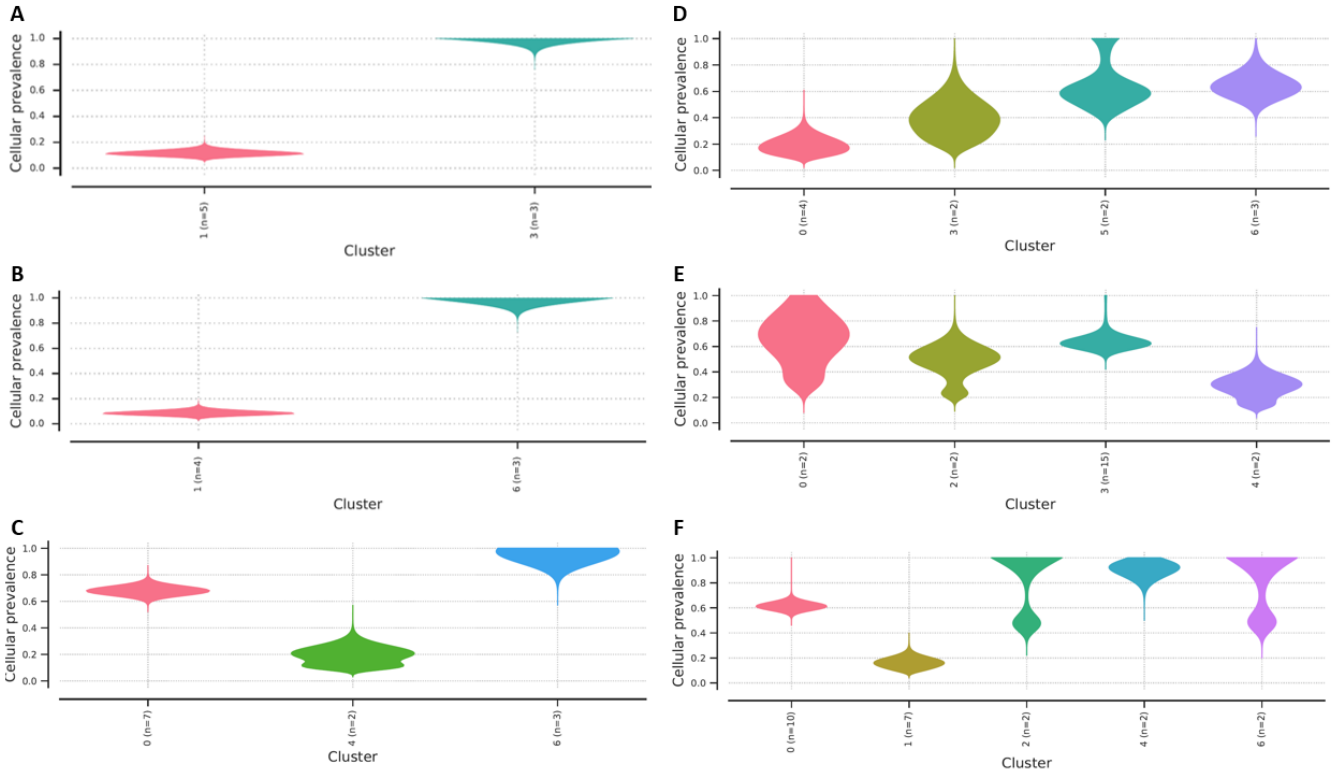

Figure S6. MRI of the neck with contrast of adenoid cystic carcinoma of right parotid gland involving masseter muscle and ascending ramus. The primary tumor and 6 subspatial regions underwent whole-exome sequencing followed by validation with deep sequencing. (a). Axial T2 MRI showing 3.8cm hyperintense right parotid mas with involvement of masseter muscle. (b). Coronal T2 MRI showing abutment against ascending ramus of mandible.

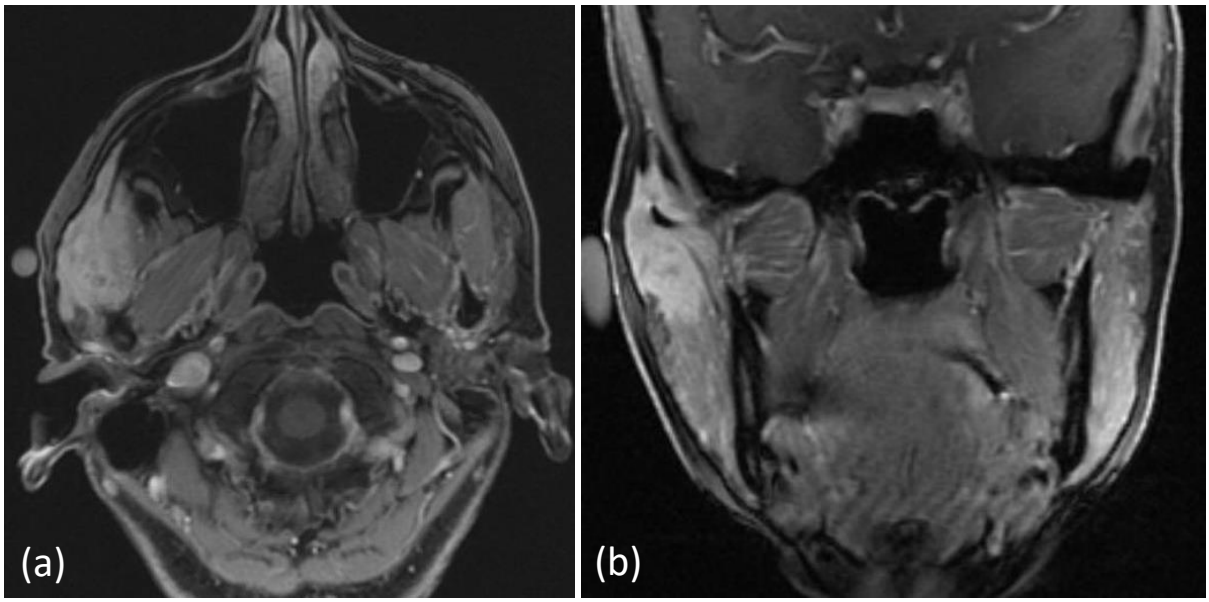

Figure S7. Histologic confirmation of 6 representative distant metastatic sites of a single case with parotid adenoid cystic carcinoma. More than 90 distant metastatic lesions were resected. Red arrows demonstrate metastatic sites. (a)Axial chest CT showing distant metastases to right upper lobe. (b) Hematoxylin and eosin (H&E) stain of Metastasis_5A. (c) H&E stain of Metastasis_6D. (d) Axial chest CT showing distant metastases to right middle lobe. (e) H&E stain of Metastasis_4A. (c) H&E stain of Metastasis_4H. (g) Axial chest CT showing distant metastases to right lower lobe. (h) H&E stain of Metastasis_4J. (i) H&E stain of Metastasis_2B.

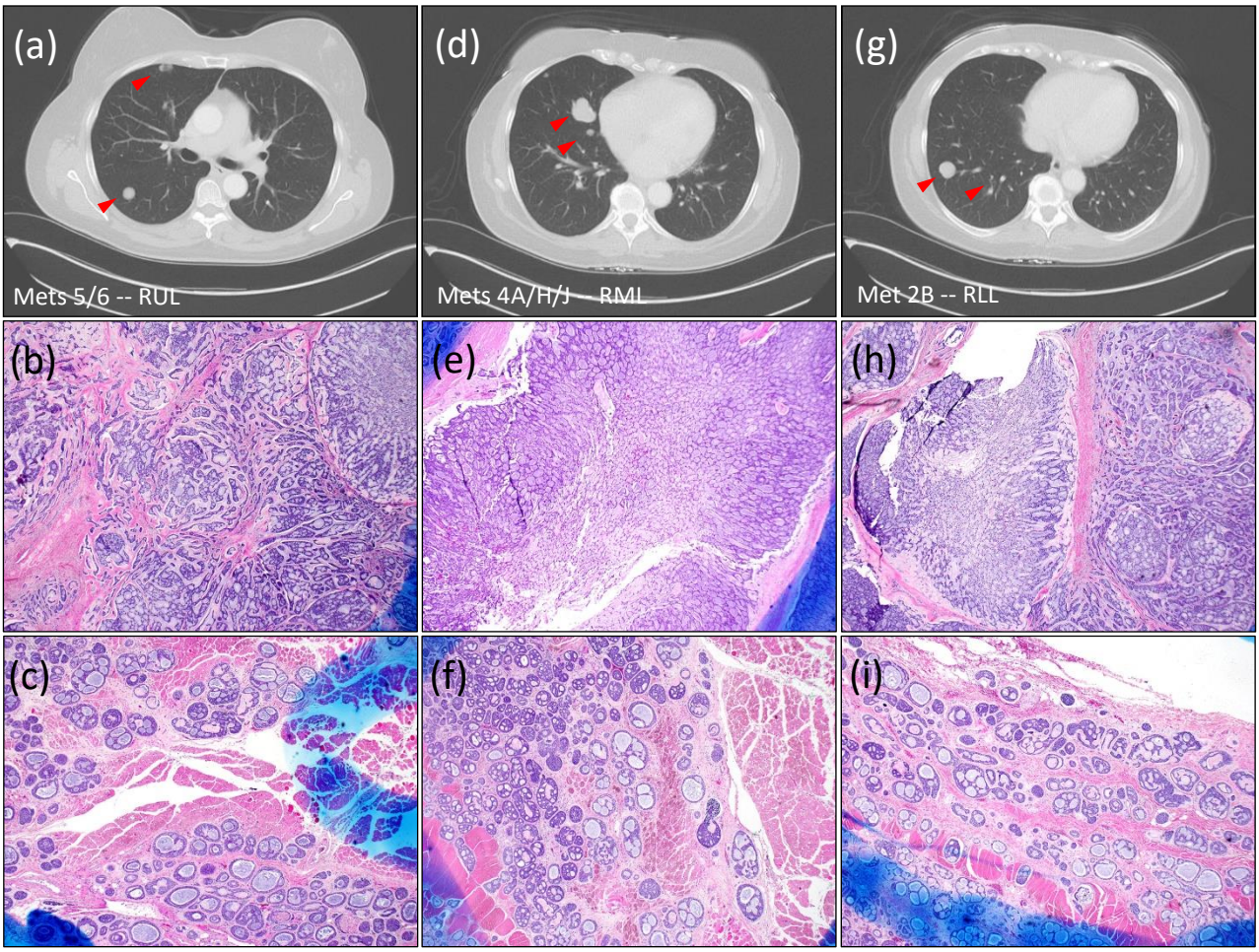

Figure S8. Fluorescence in situ hybridization (FISH) of distant lung metastatic lesions in a single case of parotid adenoid cystic carcinoma. All samples demonstrated retention of *MYB-NFIB* fusion events. FISH was performed using a three-color probe mix where green represents 5' *MYB*, orange represents 3' *MYB*, and red represents 3' *NFIB* transcripts. White arrowheads point to colocalized probes, consistent with classic *MYB-NFIB* translocation. (a). Metastatic lesion 2B. (b). Metastatic lesion 4H. (c). Metastatic lesion 4I. (d). Metastatic lesion 5C. (e). Metastatic lesion 5E. (f). Metastatic lesion 6D.

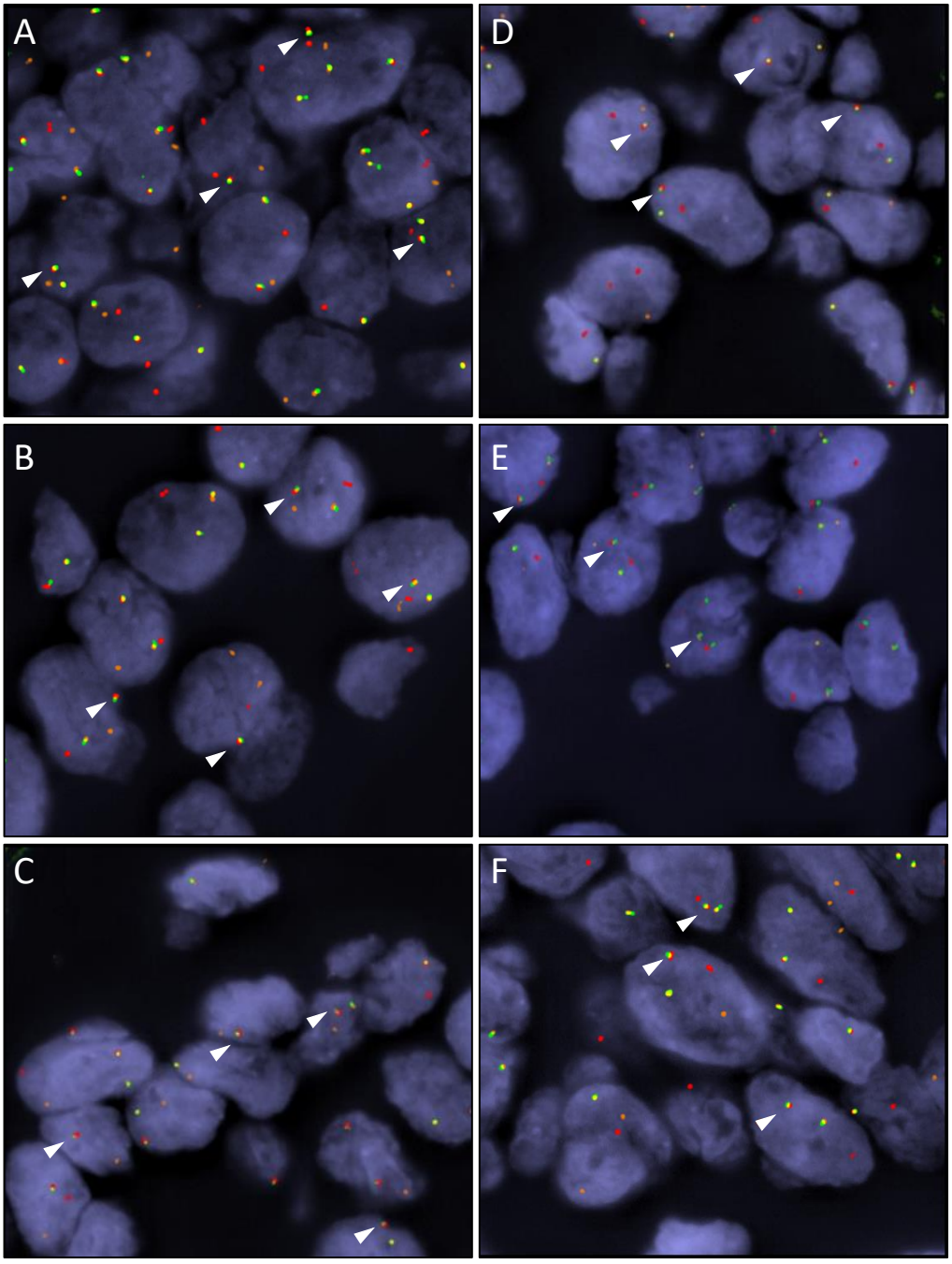

Figure S9. Two-way plots of cancer cell fraction in a single case of parotid adenoid cystic carcinoma, comparing primary tumor with eight metastatic lesions. Subgroups stratified by non-negative matrix factorization (NMF) clustering via GenePattern.

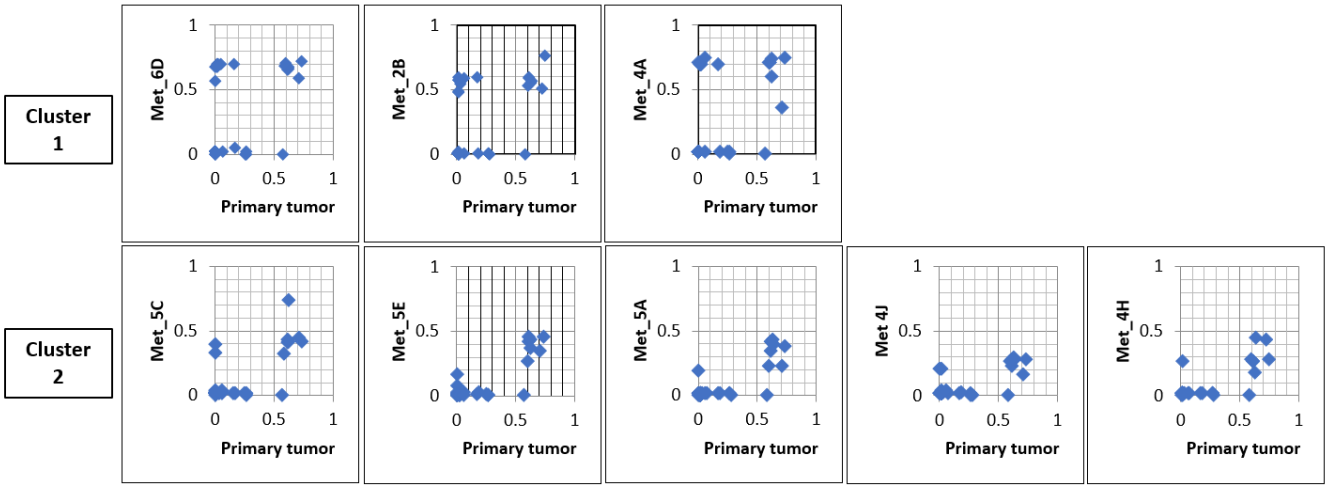

Figure S10. Multiregion clonal evolution heatmap analysis of two breast adenoid cystic carcinoma cases with transformation to high grade triple-negative breast cancer (TNBC) histology. Both cases exhibited the *MYB-NFIB* translocation throughout all multiregions analyzed. Clonality represented by cancer cell fraction in red.

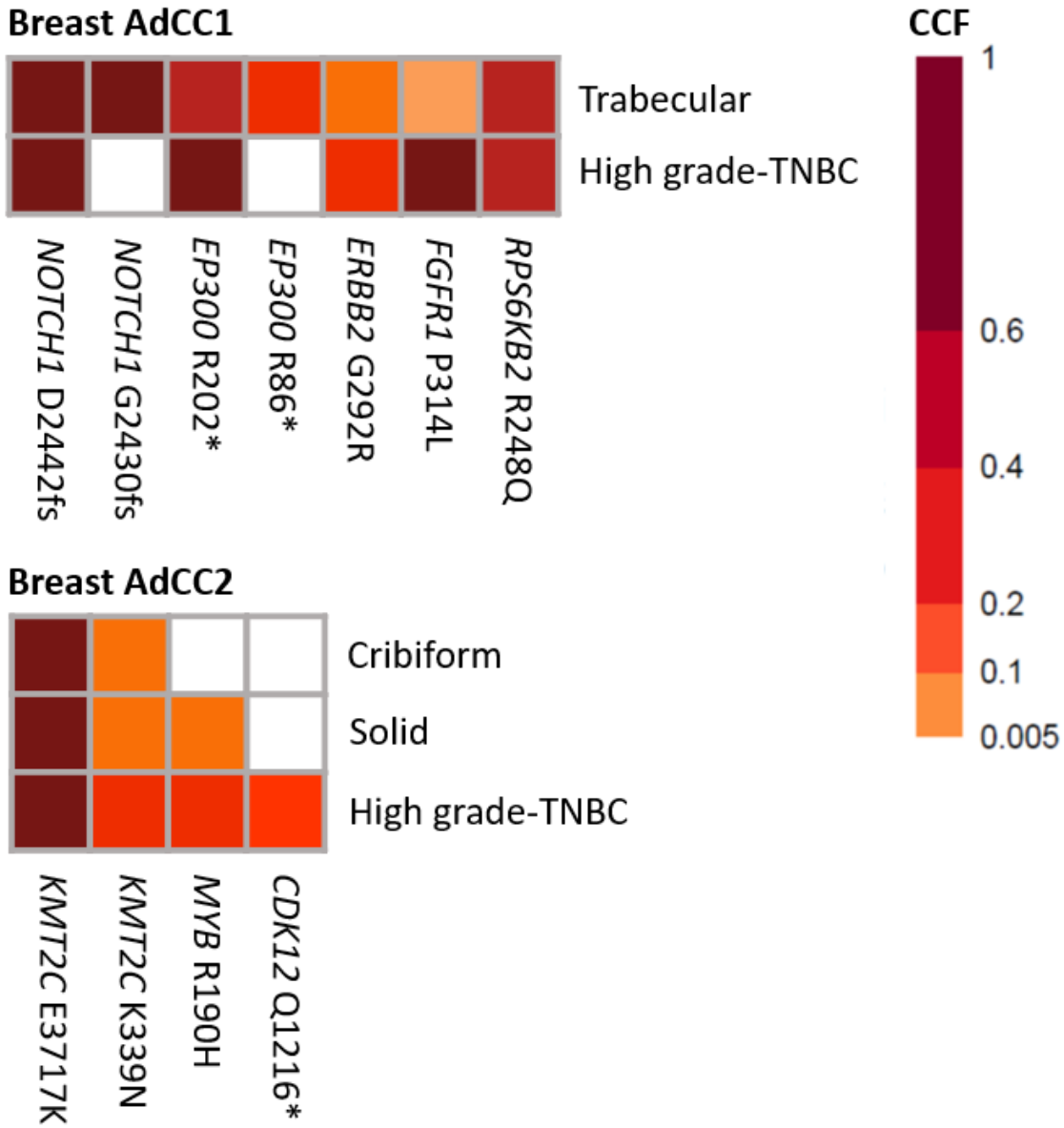

Table S1. Study distribution of primary and recurrent/metastatic (R/M) adenoid cystic carcinoma (ACC) cases. Mixed entails head and neck, lung, and breast disease sites. H&N, head and neck; WES, whole exome sequencing; WGS, whole genome sequencing; FFPE, formalin fixed paraffin embedded.

Primary ACC Studies	Institution	Site	Approach	Tissue	Matched Normal?	# Samples
Ho et al, *Nature Genetics* 2013	Memorial Sloan Kettering	H&N	WES/WGS	Fresh frozen	Yes	60
Stephens et al, *J Clin Invest* 2013	Sanger/MD Anderson	H&N	WES	Fresh frozen	Yes	24
Martelotto et al, *J Path* 2015	Memorial Sloan Kettering	Breast	WES	Fresh frozen/FFPE	Yes	12
Rettig et al, *Cancer Prev Res* 2016	Johns Hopkins	H&N	WGS	Fresh frozen	Yes	25
Mitani et al, *Clin Cancer Res* 2016	MD Anderson	H&N	WGS	Fresh frozen	Yes	21
[Sanger/MD Anderson - unpublished]	Sanger/MD Anderson	H&N	WES	Fresh frozen	Yes	35
Total						**177**

Recurrent/Metastatic ACC Studies	Institution	Site	Approach	Tissue	Matched Normal?	# Samples
Ross et al, *Am J Surg Path* 2014	Foundation Medicine	H&N	Targeted panels	FFPE	No	28
[MSKCC - unpublished]	Memorial Sloan Kettering	H&N	WES	Fresh frozen	Yes	16
[MSK-IMPACT - unpublished]	Memorial Sloan Kettering	Mixed	Targeted panels	FFPE	Yes	94
[Foundation Medicine - unpublished]	Foundation Medicine	Mixed	Targeted panels	FFPE	No	730
Total						**868**

Table S2. Top gene alteration incidence by tumor site (includes primary and recurrent/metastatic cases).

Salivary

Gene	# Alterations	Incidence
MYB	203	25.2%
NOTCH1	323	21.8%
NFIB	211	20.8%
KDM6A	138	12.9%
ARID1A	123	10.7%
KMT2C	103	10.7%
KMT2D	120	10.5%
BCOR	103	10.2%
ARID1B	74	9.2%
CREBBP	96	8.9%
TERT	76	7.5%
EP300	76	7.4%
RUNX1	75	6.8%
FAT1	54	6.7%
TP53	84	6.6%
SPEN	73	6.5%
BRCA2	59	5.9%
ATM	62	5.7%
PIK3CA	52	5.1%
PIK3R1	50	4.7%

Lung

Gene	# Alterations	Incidence
MYB	12	20.3%
NOTCH1	16	17.1%
KMT2C	16	17.0%
BCOR	13	16.0%
ARID1A	14	15.8%
ARID1B	10	15.3%
NFIB	9	11.8%
FAT1	7	10.2%
CREBBP	8	9.3%
KMT2D	8	9.3%
LRP1B	11	9.3%
TP53	10	9.2%
MED12	6	8.0%
TERT	6	7.9%
KDM6A	6	7.9%
TSC2	6	7.9%
SPEN	6	6.7%
EP300	5	6.7%
NOTCH2	5	6.7%
PTPN11	5	6.6%

Breast

Gene	# Alterations	Incidence
MYB	14	37.5%
NFIB	12	31.5%
NOTCH1	11	21.1%
CREBBP	8	18.9%
KMT2D	7	16.2%
MED12	5	13.5%
FAT3	6	11.4%
KMT2C	5	9.4%
ARID1B	3	9.4%
LRP1B	3	8.3%
NOTCH3	3	8.1%
SF3B1	3	8.1%
ARID1A	3	7.9%
KDM6A	3	7.9%
PTEN	4	7.9%
KMT2A	3	7.9%
TP53	3	7.9%
CHD2	2	6.7%
BCORL1	2	5.7%
CHEK2	2	5.3%

Table S3. Top gene alteration incidence of recurrent/metastatic adenoid cystic carcinoma (R/M ACC) cases comparing primary site with distant metastatic site.

R/M Cohort (primary tumor)

Gene	# Mut	#	Freq
NOTCH1	10	7	23.33%
NFIB	6	6	20%
KDM6A	6	6	20%
MYB	6	6	20%
ARID1A	5	5	16.67%
BCOR	4	4	13.33%
TERT	4	4	13.33%
PIK3CA	4	4	13.33%
TP53	3	3	10%
RUNX1	2	2	6.67%
CREBBP	2	2	6.67%
KMT2C	2	2	6.67%
PPP2R1A	2	2	6.67%
MTOR	2	2	6.67%
HRAS	2	2	6.67%
SMARCA4	2	2	6.67%
FGFR2	2	2	6.67%
TBX3	1	1	3.33%
RARA	1	1	3.33%
ATRX	1	1	3.33%

R/M Cohort (metastatic tumor)

Gene	# Mut	#	Freq
NFIB	20	20	29.41%
MYB	20	20	29.41%
NOTCH1	25	19	27.94%
TERT	13	13	19.12%
BCOR	9	9	13.24%
KDM6A	8	8	11.76%
TP53	9	8	11.76%
ARID1A	7	6	8.82%
KMT2D	5	5	7.35%
PIK3CA	5	5	7.35%
EP300	5	5	7.35%
RUNX1	7	4	5.88%
MGA	4	4	8.16%
ABL1	3	3	4.41%
RASA1	3	3	4.41%
IGF1R	3	3	4.41%
PTPRD	4	3	4.41%
BRCA2	3	3	4.41%
ATM	3	3	4.41%
SPEN	3	3	4.41%

Table S4. Odds ratios for top altered genes comparing primary with recurrent/metastatic (R/M) adenoid cystic carcinoma (ACC) cohorts. Nominal p-values and Benjamini-Hochberg false discovery rate (FDR)-adjusted q values are shown. *, Given that the gene panels differed for the 868 R/M cases, the denominator underlying mutation incidence is specific for each gene.

	Primary ACC (n=177)		R/M ACC (n=868*)					
Gene	# alterations	Incidence	# alterations	Incidence	Odds ratio	95% CI	p-value	BenHoch q-value
NOTCH1	15	8.5%	225	26.3%	3.86	2.23-6.70	<0.0001	0.0006
KDM6A	6	3.4%	130	15.2%	5.12	2.22-11.80	0.0001	0.0006
MLL3/KMT2C	7	4.0%	90	14.3%	4.06	1.84-8.92	0.0005	0.0012
ARID1B	7	4.0%	89	14.1%	4.00	1.82-8.81	0.0006	0.0012
ARID1A	4	2.3%	117	13.7%	6.87	2.50-18.86	0.0002	0.0006
BCOR	3	1.7%	107	13.3%	8.92	2.80-28.42	0.0002	0.0006
MLL2/KMT2D	8	4.5%	103	12.8%	3.10	1.48-6.50	0.0027	0.0046
CREBBP	8	4.5%	89	11.1%	2.63	1.25-5.53	0.011	0.013
EP300	5	2.8%	73	9.1%	3.44	1.37-8.64	0.0086	0.013
RUNX1	5	2.8%	68	8.0%	2.98	1.18-7.49	0.021	0.021
LRP1B	2	1.1%	51	6.8%	6.43	1.55-26.67	0.010	0.013
ATM	3	1.7%	56	6.8%	4.22	1.31-13.63	0.016	0.017

Table S5. Variant allele fraction (VAF) for most commonly mutated genes in recurrent/metastatic adenoid cystic carcinoma (R/M ACC).

R/M ACC Cases

Key genes	# point mutations	Average VAF	# cases w/VAF <0.05	% cases w/VAF <0.05
NOTCH1	337	0.36	12	3.6%
KDM6A	141	0.41	0	0.0%
ARID1A	136	0.32	4	2.9%
MLL2/KMT2D	125	0.36	0	0.0%
BCOR	116	0.39	0	0.0%
MLL3/KMT2C	115	0.29	1	0.9%
CREBBP	102	0.29	5	4.9%
ARID1B	85	0.37	0	0.0%
TERT	83	0.37	0	0.0%
EP300	77	0.30	2	2.6%
RUNX1	73	0.25	3	4.1%
ATM	63	0.35	3	4.8%
LRP1B	57	0.44	1	1.8%
NOTCH3	47	0.42	0	0.0%
NOTCH2	37	0.39	1	2.7%
NOTCH4	27	0.43	0	0.0%

Table S6. Downsampling analysis of R/M MSKCC-IMPACT cohort. Five independently downsampled BAM files generated at 100x coverage (original BAM files at 600x), with reads randomly selected during downsampling. Only one mutation (red highlight) did not pass filters and would have been missed at 100x coverage.

DMP_ID	Gene	Total depth	Alt Depth	IMPACT VAF	Downsampled BAM1 VAF	Downsampled BAM2 VAF	Downsampled BAM3 VAF	Downsampled BAM4 VAF	Downsampled BAM5 VAF	Downsampled BAM Ave VAF
P0006690-T01-IM5	MLL2	93	36	0.3871	0.47059	0.41463	0.34884	0.35294	0.35	0.3874
P0011474-T01-IM5	BCOR	876	698	0.7968	0.76786	0.81967	0.875	0.76271	0.92647	0.830342
P0014709-T01-IM6	ATM	736	57	0.07745	0.07595	0.08824	0.07042	0.03947	0.04938	0.064692
P0012051-T01-IM5	ARID1A	801	75	0.09363	0.17333	0.08421	0.10145	0.05172	0.0625	0.094642
P0011474-T01-IM5	EP300	987	229	0.23202	0.23077	0.3271	0.2126	0.23656	0.30337	0.26208
P0009457-T01-IM5	ARID1A	977	686	0.70215	0.69565	0.63636	0.63551	0.69697	0.80435	0.693768
P0000790-T01-IM3	RUNX1	221	47	0.21267	0.18667	0.26761	0.22973	0.35135	0.27778	0.262628
P0011474-T01-IM5	RUNX1	218	18	0.08257	0.09859	0.06944	0.09375	0.11268	0.10145	0.095182
P0011474-T01-IM5	NOTCH1	156	69	0.44231	0.56522	0.53571	0.32653	0.2963	0.51786	0.448324
P0001201-T01-IM3	BCOR	607	219	0.36079	0.35294	0.42308	0.32381	0.31034	0.42727	0.367488
P0014472-T01-IM6	NOTCH1	321	93	0.28972	0.40698	0.37333	0.3956	0.32911	0.32	0.365004
P0013084-T01-IM5	NOTCH1	371	147	0.39623	0.43038	0.35106	0.46237	0.36709	0.51765	0.42571
P0000623-T01-IM3	RUNX1	191	13	0.06806	0.07143	0.04255	0.13793	0.12069	0.02083	0.078686
P0012051-T01-IM5	EP300	802	292	0.36409	0.34286	0.39024	0.26027	0.27381	0.44186	0.341808
P0001422-T01-IM3	ARID1B	906	446	0.49227	0.45455	0.45631	0.40789	0.39048	0.55789	0.453424
P0014472-T01-IM6	NOTCH1	833	334	0.40096	0.5	0.55789	0.4881	0.53333	0.40698	0.49726
P0014382-T01-IM6	NOTCH1	1198	498	0.41569	0.46226	0.48855	0.39837	0.4661	0.45631	0.454318
P0000980-T01-IM3	NOTCH1	102	56	0.54902	0.48649	0.44828	0.51724	0.46154	0.57692	0.498094
P0009317-T02-IM5	NOTCH1	143	90	0.62937	0.63889	0.4918	0.7	0.73171	0.58	0.62848
P0014382-T01-IM6	BCOR	228	76	0.33333	0.41791	0.35593	0.25	0.3125	0.42029	0.351326
P0002214-T01-IM3	MLL3	246	19	0.07724	0.13462	0.02564	0.13514	0.13158	0.14	0.113396
P0000948-T01-IM3	**NOTCH1**	**375**	**20**	**0.05333**	0.01562	0	0.05797	0.05634	0.06944	0.039874
P0014709-T01-IM6	BCOR	276	196	0.71014	0.75	0.76562	0.75	0.70175	0.75472	0.744418
P0014709-T01-IM6	ATM	480	159	0.33125	0.37255	0.33333	0.35461	0.31507	0.36	0.347112
P0011474-T01-IM5	ARID1A	211	123	0.58294	0.59091	0.57353	0.60811	0.53226	0.625	0.585962
P0002486-T01-IM3	NOTCH1	284	111	0.39085	0.32258	0.3	0.27473	0.36923	0.36364	0.326036
P0001585-T01-IM3	NOTCH1	101	23	0.22772	0.24242	0.32143	0.15625	0.15909	0.23529	0.222896
P0014709-T01-IM6	KDM6A	381	152	0.39895	0.38318	0.47273	0.42623	0.37624	0.41803	0.415282
P0012652-T01-IM5	KDM6A	1065	299	0.28075	0.27273	0.32381	0.31061	0.2193	0.20472	0.266234
P0000340-T01-IM3	BCOR	504	302	0.59921	0.48	0.64151	0.45714	0.57447	0.76667	0.583958
P0008688-T01-IM5	RUNX1	777	277	0.3565	0.31122	0.32618	0.4	0.3299	0.36715	0.34689

P0001451-T01-IM3	KDM6A	261	33	0.12644	0.12712	0.18182	0.13889	0.128	0.14851	0.144868
P0008768-T01-IM5	ARID1A	132	38	0.28788	0.46429	0.53333	0.6087	0.65217	0.56	0.563698
P0000340-T01-IM3	NOTCH1	199	69	0.34673	0.31707	0.42857	0.27586	0.28889	0.38095	0.338268
P0006518-T01-IM5	KDM6A	412	232	0.56311	0.61446	0.53247	0.62162	0.53488	0.53488	0.567662
P0000507-T01-IM3	RUNX1	511	27	0.05284	0.05455	0.02985	0.07692	0.11864	0.03448	0.062888
P0001201-T01-IM3	RUNX1	869	172	0.19793	0.13095	0.15	0.27193	0.17757	0.20792	0.187674
P0010663-T01-IM5	RUNX1	699	91	0.13019	0.17722	0.15714	0.11538	0.14607	0.15584	0.15033
P0001422-T01-IM3	EP300	949	147	0.1549	0.14286	0.1039	0.10204	0.18812	0.1913	0.145644
P0017600-T01-IM5	MLL2	622	46	0.07395	0.05983	0.11	0.06087	0.11111	0.05941	0.080244
P0000792-T01-IM3	NOTCH1	725	164	0.22621	0.31452	0.27679	0.25	0.19828	0.2619	0.260298
P0005624-T02-IM5	BCOR	761	265	0.34823	0.36066	0.30693	0.36134	0.35	0.34513	0.344812
P0007499-T01-IM5	MLL2	395	190	0.48101	0.51724	0.39474	0.59524	0.47458	0.54545	0.50545
P0002486-T01-IM3	KDM6A	844	700	0.82938	0.9322	0.92784	0.83784	0.90291	0.90566	0.90129
P0002486-T01-IM3	NOTCH1	684	473	0.69152	0.64423	0.73333	0.7125	0.62338	0.71717	0.686122
P0001585-T01-IM3	BCOR	252	173	0.68651	0.65625	0.5	0.71429	0.65	0.70968	0.646044
P0001451-T01-IM3	CREBBP	146	23	0.15753	0.13699	0.22807	0.2	0.19753	0.15625	0.183768
P0007499-T01-IM5	NOTCH1	123	36	0.29268	0.34545	0.24561	0.31148	0.27273	0.28571	0.292196
P0013838-T01-IM5	NOTCH1	160	49	0.30625	0.31395	0.31707	0.33735	0.24444	0.27536	0.297634
P0000618-T01-IM3	NOTCH1	1140	894	0.78421	0.77612	0.76496	0.73478	0.75336	0.85306	0.776456
P0005392-T01-IM5	ARID1A	788	323	0.4099	0.43902	0.45946	0.5098	0.44118	0.38889	0.44767
P0007145-T01-IM5	ARID1A	675	266	0.39407	0.42718	0.40678	0.36029	0.45161	0.39695	0.408562
P0001201-T01-IM3	EP300	700	276	0.39429	0.38542	0.328	0.39516	0.36082	0.47541	0.388962
P0000434-T01-IM3	NOTCH1	607	454	0.74794	0.81013	0.73034	0.76289	0.65591	0.72277	0.736408
P0001451-T01-IM3	BCOR	586	242	0.41297	0.44681	0.42268	0.42593	0.36842	0.53623	0.440014
P0003649-T01-IM5	KDM6A	1756	418	0.23804	0.22798	0.29353	0.2201	0.27273	0.27273	0.257414
P0000623-T02-IM5	ARID1A	549	208	0.37887	0.38686	0.40789	0.37589	0.3662	0.45	0.397368
P0000374-T01-IM3	KDM6A	462	34	0.07359	0.09735	0.08257	0.09322	0.07826	0.12069	0.094418
P0015401-T01-IM6	ARID1A	497	169	0.34004	0.33051	0.35606	0.32593	0.36879	0.3871	0.353678
P0000340-T01-IM3	BCOR	670	336	0.50149	0.53922	0.44118	0.39423	0.56098	0.46429	0.47998
P0003327-T01-IM5	NOTCH1	651	78	0.11982	0.06667	0.10744	0.15789	0.15556	0.06306	0.110124
P0003056-T01-IM5	BCOR	497	249	0.50101	0.66667	0.69444	0.73134	0.71831	0.59211	0.680574
P0014961-T01-IM6	RUNX1	165	37	0.22424	0.21296	0.21239	0.23577	0.24	0.21138	0.2225
P0000623-T01-IM3	ARID1A	150	23	0.15333	0.1875	0.38462	0.36	0.52	0.16667	0.323758
P0014961-T01-IM6	BCOR	886	73	0.08239	0.13768	0.152	0.18045	0.25333	0.2129	0.187272
P0001451-T01-IM3	RUNX1	635	79	0.12441	0.11765	0.14563	0.11765	0.09184	0.16814	0.128182

P0000374-T01-IM3	NOTCH1	1347	1102	0.81811	0.84615	0.79545	0.84932	0.72159	0.79141	0.800784
P0015101-T01-IM6	NOTCH1	1416	680	0.48023	0.49091	0.49405	0.46707	0.44693	0.42778	0.465348
P0014961-T01-IM6	MLL2	506	183	0.36166	0.46154	0.35135	0.37975	0.34286	0.34444	0.375988
P0001422-T01-IM3	NOTCH1	318	177	0.5566	0.49091	0.7234	0.4	0.46512	0.48333	0.512552
P0007145-T01-IM5	KDM6A	779	218	0.27985	0.23457	0.2381	0.24286	0.33333	0.3	0.269772
P0007145-T01-IM5	ATM	774	68	0.08786	0.15714	0.07246	0.12195	0.1	0.06098	0.102506
P0004371-T01-IM5	KDM6A	689	63	0.09144	0.075	0.07895	0.08451	0.06557	0.10448	0.081702
P0013838-T01-IM5	NOTCH1	951	365	0.38381	0.28571	0.31496	0.36752	0.38519	0.34188	0.339052
P0006032-T01-IM5	KDM6A	465	392	0.84301	0.83673	0.81818	0.7971	0.87179	0.8	0.82476
P0016400-T01-IM6	KDM6A	293	181	0.61775	0.55769	0.62069	0.57143	0.72857	0.64474	0.624624
P0007145-T01-IM5	ATM	391	24	0.06138	0.03448	0.08571	0.05769	0.05882	0.05	0.05734
P0000524-T01-IM3	MLL2	166	13	0.07831	0.13636	0.09091	0	0.05556	0.11765	0.080096
P0000202-T01-IM3	MLL3	1145	69	0.06026	0.09722	0.05806	0.10317	0.02857	0.04444	0.066292
P0003327-T01-IM5	NOTCH1	858	154	0.17949	0.14953	0.18487	0.13187	0.16304	0.24444	0.17475
P0000618-T01-IM3	BCOR	598	58	0.09699	0.03333	0.10989	0.07812	0.08333	0.08989	0.078912
P0000618-T01-IM3	NOTCH1	602	58	0.09635	0.10526	0.04225	0.04918	0.11842	0.12281	0.087584
P0012652-T01-IM5	CREBBP	916	420	0.45852	0.45882	0.53097	0.42574	0.50877	0.47368	0.479596
P0007499-T01-IM5	NOTCH1	731	178	0.2435	0.15476	0.24051	0.28571	0.36264	0.24286	0.257296
P0009174-T01-IM5	ARID1A	1133	320	0.28244	0.35211	0.29323	0.27273	0.21429	0.27152	0.280776
P0001451-T01-IM3	ARID1B	864	267	0.30903	0.31868	0.39604	0.37895	0.21495	0.34615	0.330954
P0007849-T02-IM5	KDM6A	533	305	0.57223	0.55385	0.60294	0.56452	0.5873	0.60294	0.58231
P0000524-T01-IM3	NOTCH1	1277	276	0.21613	0.24812	0.22137	0.21094	0.18898	0.24818	0.223518
P0012563-T01-IM5	NOTCH1	1188	211	0.17761	0.24812	0.19841	0.23188	0.12698	0.176	0.196278
P0000980-T01-IM3	NOTCH1	845	590	0.69822	0.65476	0.72941	0.70423	0.72043	0.69149	0.700064
P0012563-T01-IM5	KDM6A	729	465	0.63786	0.5	0.69565	0.62687	0.36842	0.66234	0.570656
P0008066-T01-IM5	BCOR	521	32	0.06142	0.11594	0.08642	0.02667	0.05495	0.06522	0.06984
P0012652-T01-IM5	NOTCH1	733	213	0.29059	0.27778	0.34722	0.35366	0.37931	0.30233	0.33206
P0014961-T01-IM6	NOTCH1	819	369	0.45055	0.54054	0.41975	0.50685	0.42391	0.44444	0.467098
P0006690-T01-IM5	CREBBP	1952	246	0.12602	0.13298	0.14205	0.09787	0.11312	0.09205	0.115614
P0014405-T01-IM6	KDM6A	1023	123	0.12023	0.0885	0.1028	0.09474	0.14516	0.09615	0.10547
P0004371-T01-IM5	EP300	902	257	0.28492	0.24706	0.30208	0.23529	0.27397	0.25806	0.263292
P0005392-T01-IM5	NOTCH1	696	635	0.91236	0.93162	0.89362	0.90741	0.91667	0.93636	0.917136
P0010654-T01-IM5	ARID1A	425	93	0.21882	0.33036	0.15152	0.24771	0.25	0.232	0.242318
P0000202-T01-IM3	NOTCH1	622	222	0.35691	0.31481	0.40909	0.38889	0.4	0.36646	0.37585
P0019072-T01-IM6	NOTCH1	533	118	0.22139	0.17857	0.21477	0.27642	0.22901	0.20968	0.22169

Table S7. Pyclone subclonal population analysis of 58 adenoid cystic carcinoma patients. A tumor was considered subclonal if it comprised at least 2 clusters (each with a minimum of 2 mutations), with at least one cluster having an upper 95% confidence interval (CI) below 0.95. SCP, subclonal population.

ID	Cohort	Subclonal?	# SCP	# Clusters	Upper bound 95% CI
05_6986	Primary	yes	2	2 clusters	0.61
06_2532	Primary	no		2 clusters	0.98
07_16582	Primary	no		2 clusters	1.06
09_4178	Primary	yes	2	2 clusters	0.63
09_4615	Primary	no		1 cluster	0.91
10_5283	Primary	no		2 clusters	0.98
11_3318	Primary	yes	2	2 clusters	0.78
11_6165	Primary	yes	2	2 clusters	0.47
11_17815	Primary	no		1 cluster	1.00
236	Primary	no		1 cluster	0.71
540	Primary	no		1 cluster	0.75
609	Primary	no		1 cluster	0.65
705	Primary	no		1 cluster	0.90
1346	Primary	no		1 cluster	0.28
1739	Primary	no		1 cluster	0.79
1781	Primary	no		1 cluster	1.05
1947	Primary	no		2 clusters	1.01
2039	Primary	yes	2	2 clusters	0.73
2237	Primary	no		1 cluster	1.02
2238	Primary	no		1 cluster	1.08
3492	Primary	yes	2	2 clusters	0.22
4133	Primary	yes	2	2 clusters	0.42
6277	Primary	no		1 cluster	0.73
7097	Primary	no		1 cluster	0.53
7136	Primary	yes	2	2 clusters	0.88
7441	Primary	no		1 cluster	1.04
9534	Primary	yes	2	2 clusters	0.34
65115	Primary	no		1 cluster	0.77
80872	Primary	no		1 cluster	0.76

ID	Cohort	Subclonal?	# SCP	# Clusters	Upper bound 95% CI
111097	Primary	no		1 cluster	1.15
118135	Primary	yes	2	2 clusters	0.62
122891	Primary	no		1 cluster	0.95
142990	Primary	no		1 cluster	0.49
148632	Primary	yes	3	3 clusters	0.36
671204	Primary	yes	2	2 clusters	0.34
980452	Primary	no		1 cluster	0.26
990149	Primary	no		1 cluster	0.36
2000120	Primary	yes	2	2 clusters	0.71
2000136	Primary	no		1 cluster	0.55
2000756	Primary	no		1 cluster	0.51
36773720	Primary	yes	4	4 clusters	0.58
YLE001	Primary	yes	2	2 clusters	N/A
C2725	R/M	No		1 cluster	1.20
C2954	R/M	No		1 cluster	1.05
C3070	R/M	Yes	5	5 clusters	0.45
D3212	R/M	Yes	4	4 clusters	0.45
F0975	R/M	No		1 cluster	1.05
F2608	R/M	Yes	2	2 clusters	0.31
F6345	R/M	No		1 cluster	0.41
G3856	R/M	Yes	2	2 clusters	0.92
H1407	R/M	No		2 clusters	1.04
H1985	R/M	No		2 clusters	1.12
K8414	R/M	No		1 cluster	0.67
M9671	R/M	Yes	2	2 clusters	0.23
P1849	R/M	No		1 cluster	1.03
T0669	R/M	No		1 cluster	1.06
W7869	R/M	No		1 cluster	0.72
W9012	R/M	No		1 cluster	0.36

Table S8. Pathogenic germline variants detected in recurrent/metastatic adenoid cystic carcinoma.

Sample	Chr	Start	Ref	Alt	VariantClass	Gene	Exon	TranscriptID	cDNAchange	Penetrance	Type	ACMG Category
Sample40	5	112162961	T	C	splicing	*APC*	exon12	NM_000038	c.1548+17T>C	low		Pathogenic
Sample16	17	41215871	A	T	splicing	*BRCA1*	exon18	NM_007294	c.5152+20T>A	high	DNA repair	Pathogenic
Sample29	17	41201130	A	G	splicing	*BRCA1*	exon22	NM_007294	c.5406+8T>C	high	DNA repair	Pathogenic
Sample32	17	41203077	T	C	splicing	*BRCA1*	exon21	NM_007294	c.5332+3A>G	high	DNA repair	Pathogenic
Sample51	13	32968810	T	C	splicing	*BRCA2*	exon25	NM_000059	c.9257-16T>C	high	DNA repair	Pathogenic
Sample02	16	68855885	T	C	splicing	*CDH1*	exon12	NM_004360	c.1712-19T>C	high		Pathogenic
Sample84	16	68842578	C	T	splicing	*CDH1*	exon5	NM_004360	c.532-18C>T	high		Pathogenic
Sample59	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample64	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample64	3	37070265	T	G	splicing	*MLH1*	exon13	NM_000249	c.1410-10T>G	high	DNA repair	Pathogenic
Sample75	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample79	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample20	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample35	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample40	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample41	3	37067120	T	A	splicing	*MLH1*	exon12	NM_000249	c.1039-8T>A	high	DNA repair	Pathogenic
Sample86	2	48033981	T	TTTGA	FS_insertion	*MSH6*	exon10	NM_000179	c.4065_4066insTTGA	high	DNA repair	Pathogenic
Sample36	2	48032033	C	T	splicing	*MSH6*	exon6	NM_000179	c.3439-16C>T	high	DNA repair	Pathogenic
Sample42	2	48028314	T	C	splicing	*MSH6*	exon4	NM_000179	c.3172+20T>C	high	DNA repair	Pathogenic
Sample58	2	48033514	T	C	splicing	*MSH6*	exon8	NM_000179	c.3801+17T>C	high	DNA repair	Pathogenic
Sample63	2	48033898	T	G	splicing	*MSH6*	exon10	NM_000179	c.4002-20T>G	high	DNA repair	Pathogenic
Sample76	2	48028314	T	C	splicing	*MSH6*	exon4	NM_000179	c.3172+20T>C	high	DNA repair	Pathogenic
Sample30	2	48033981	T	TTTGA	FS_insertion	*MSH6*	exon10	NM_000179	c.4065_4066insTTGA	high	DNA repair	Pathogenic
Sample20	17	29679439	T	C	splicing	*NF1*	exon51	NM_001042492	c.7615+7T>C	high		Pathogenic
Sample24	17	29587544	C	G	splicing	*NF1*	exon34	NM_001042492	c.4577+11C>G	high		Pathogenic
Sample26	22	30000121	G	T	splicing	*NF2*	exon1	NM_000268	c.114+20G>T			Pathogenic
Sample43	22	30064307	C	T	splicing	*NF2*	exon10	NM_000268	c.886-15C>T			Pathogenic
Sample67	10	89720633	C	CTTT	splicing	*PTEN*	exon8	NM_000314	c.802-18->TTT			Pathogenic
Sample12	13	48934275	A	G	splicing	*RB1*	exon7	NM_000321	c.718+12A>G			Pathogenic
Sample51	13	49050826	G	A	splicing	*RB1*	exon25	NM_000321	c.2521-11G>A			Pathogenic
Sample64	13	49039118	T	A	splicing	*RB1*	exon22	NM_000321	c.2212-16T>A			Pathogenic
Sample18	5	225697	G	C	splicing	*SDHA*	exon4	NM_004168	c.456+20G>C	high		Pathogenic
Sample45	5	225515	G	T	splicing	*SDHA*	exon4	NM_004168	c.313-19G>T	high		Pathogenic
Sample80	5	225697	G	C	splicing	*SDHA*	exon4	NM_004168	c.456+20G>C	high		Pathogenic
Sample23	11	61205337	C	CTT	splicing	*SDHAF2*	exon2	NM_017841	c.260+17->TT			Pathogenic
Sample50	1	17354373	G	GGAAGAA	splicing	*SDHB*	exon6	NM_003000	c.424-13->TTCTTC	high		Pathogenic
Sample80	1	17355075	A	T	splicing	*SDHB*	exon5	NM_003000	c.423+20T>A	high		Pathogenic
Sample83	1	17355075	A	T	splicing	*SDHB*	exon5	NM_003000	c.423+20T>A	high		Pathogenic

Table S9. BRCA1/BRCA2 second hit analysis.

Sample	Chr	Variant Class	Gene	Exon	cDNA	N_Total Depth	N_Ref Count	N_Alt Count	N_Alt Freq	Tumour AlleleStatus	Somatic BRCA Variant
Sample16	17	splicing	BRCA1	exon18	c.5152+20T>A	574	324	250	0.43554	Diploid	None
Sample32	17	splicing	BRCA1	exon21	c.5332+3A>G	649	325	324	0.49923	Diploid	None
Sample29	17	splicing	BRCA1	exon22	c.5406+8T>C	586	286	300	0.51195	Gain	None
Sample51	13	splicing	BRCA2	exon25	c.9257-16T>C	324	162	162	0.5	Diploid	None

Table S10. MSISensor score for MSK-IMPACT recurrent/metastatic adenoid cystic carcinoma cases.

No.	Sample ID	Patient ID	MSI Score
1	P-0007699-T02-IM5	P-0007699	3.87
2	P-0007849-T01-IM5	P-0007849	3.45
3	P-0019072-T01-IM6	P-0019072	2.83
4	P-0013084-T01-IM5	P-0013084	2.67
5	P-0008688-T01-IM5	P-0008688	2.35
6	P-0014405-T01-IM6	P-0014405	2.15
7	P-0007849-T02-IM5	P-0007849	2.03
8	P-0004371-T01-IM5	P-0004371	2.02
9	P-0003469-T01-IM5	P-0003469	1.97
10	P-0000948-T01-IM3	P-0000948	1.61
11	P-0001327-T01-IM3	P-0001327	1.38
12	P-0007145-T01-IM5	P-0007145	0.95
13	P-0007096-T01-IM5	P-0007096	0.88
14	P-0004334-T01-IM5	P-0004334	0.78
15	P-0005392-T01-IM5	P-0005392	0.77
16	P-0017244-T01-IM6	P-0017244	0.72
17	P-0012051-T01-IM5	P-0012051	0.71
18	P-0015992-T01-IM6	P-0015992	0.67
19	P-0006939-T01-IM5	P-0006939	0.66
20	P-0007777-T01-IM5	P-0007777	0.65
21	P-0000374-T01-IM3	P-0000374	0.61
22	P-0003532-T02-IM5	P-0003532	0.6
23	P-0008066-T01-IM5	P-0008066	0.58
24	P-0003056-T01-IM5	P-0003056	0.58
25	P-0010654-T01-IM5	P-0010654	0.53
26	P-0001201-T01-IM3	P-0001201	0.47
27	P-0000717-T01-IM3	P-0000717	0.46
28	P-0012652-T01-IM5	P-0012652	0.43
29	P-0012563-T01-IM5	P-0012563	0.43
30	P-0002486-T01-IM3	P-0002486	0.37
31	P-0002214-T01-IM3	P-0002214	0.37
32	P-0000524-T01-IM3	P-0000524	0.37
33	P-0000340-T01-IM3	P-0000340	0.37
34	P-0000202-T01-IM3	P-0000202	0.34
35	P-0017620-T01-IM5	P-0017620	0.33
36	P-0001363-T01-IM3	P-0001363	0.33
37	P-0000623-T01-IM3	P-0000623	0.33
38	P-0009832-T01-IM5	P-0009832	0.32
39	P-0008493-T02-IM5	P-0008493	0.31
40	P-0002189-T01-IM3	P-0002189	0.3
41	P-0001585-T01-IM3	P-0001585	0.3
42	P-0001422-T01-IM3	P-0001422	0.29
43	P-0009317-T02-IM5	P-0009317	0.27
44	P-0015093-T01-IM6	P-0015093	0.26
45	P-0014961-T01-IM6	P-0014961	0.26
46	P-0006032-T01-IM5	P-0006032	0.26
47	P-0016362-T01-IM6	P-0016362	0.25
48	P-0008768-T01-IM5	P-0008768	0.25
49	P-0008385-T01-IM5	P-0008385	0.24
50	P-0003327-T01-IM5	P-0003327	0.24
51	P-0017600-T01-IM5	P-0017600	0.23
52	P-0017745-T01-IM6	P-0017745	0.22
53	P-0014382-T01-IM6	P-0014382	0.2
54	P-0007699-T01-IM5	P-0007699	0.17
55	P-0001660-T01-IM3	P-0001660	0.17
56	P-0001239-T01-IM3	P-0001239	0.17
57	P-0001034-T01-IM3	P-0001034	0.17
58	P-0000790-T01-IM3	P-0000790	0.17
59	P-0000507-T01-IM3	P-0000507	0.16
60	P-0000434-T01-IM3	P-0000434	0.11
61	P-0003649-T01-IM5	P-0003649	0.1
62	P-0008151-T01-IM5	P-0008151	0.09
63	P-0001451-T01-IM3	P-0001451	0.09
64	P-0009457-T01-IM5	P-0009457	0.08
65	P-0009174-T01-IM5	P-0009174	0.08
66	P-0003699-T01-IM5	P-0003699	0.08
67	P-0000618-T01-IM3	P-0000618	0.08
68	P-0016421-T01-IM6	P-0016421	0.07
69	P-0013620-T01-IM5	P-0013620	0.07
70	P-0005624-T02-IM5	P-0005624	0.07
71	P-0018440-T01-IM6	P-0018440	0
72	P-0015401-T01-IM6	P-0015401	0
73	P-0016400-T01-IM6	P-0016400	0
74	P-0015101-T01-IM6	P-0015101	0
75	P-0014709-T01-IM6	P-0014709	0
76	P-0014472-T01-IM6	P-0014472	0
77	P-0013838-T01-IM5	P-0013838	0
78	P-0011474-T01-IM5	P-0011474	0
79	P-0010663-T01-IM5	P-0010663	0
80	P-0009534-T01-IM5	P-0009534	0
81	P-0008227-T01-IM5	P-0008227	0
82	P-0008045-T01-IM5	P-0008045	0
83	P-0007857-T01-IM5	P-0007857	0
84	P-0007499-T01-IM5	P-0007499	0
85	P-0007102-T01-IM5	P-0007102	0
86	P-0006690-T01-IM5	P-0006690	0
87	P-0006518-T01-IM5	P-0006518	0
88	P-0005382-T01-IM5	P-0005382	0
89	P-0004887-T01-IM5	P-0004887	0
90	P-0004186-T01-IM5	P-0004186	0
91	P-0003532-T01-IM5	P-0003532	0
92	P-0003111-T01-IM5	P-0003111	0
93	P-0001810-T01-IM3	P-0001810	0
94	P-0001225-T01-IM3	P-0001225	0
95	P-0001220-T01-IM3	P-0001220	0
96	P-0000980-T01-IM3	P-0000980	0
97	P-0000792-T01-IM3	P-0000792	0
98	P-0000623-T02-IM5	P-0000623	0

The REMARK checklist

	Item to be reported	Page no.
INTRODUCTION		
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	1
MATERIALS AND METHODS		
Patients		
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	18
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	18
Specimen characteristics		
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	18
Assay methods		
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	18
Study design		
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	18
7	Precisely define all clinical endpoints examined.	18
8	List all candidate variables initially examined or considered for inclusion in models.	18
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	18
Statistical analysis methods		
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	23
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	19
RESULTS		
Data		
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	18
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	35
Analysis and presentation		
14	Show the relation of the marker to standard prognostic variables.	38
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	38
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	n/a
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	n/a
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	5
DISCUSSION		
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	11
20	Discuss implications for future research and clinical value.	11

Source: McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM: Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005; 97: 1180-1184.

INTRODUCTION

1. State the marker examined, the study objectives, and any pre-specified hypotheses.

Using sequencing data from 1043 adenoid cystic carcinoma patients (ACC), we investigated the genomic differences between primary ACC and recurrent/metastatic (R/M) ACC.

The objective of the study was to evaluate the underlying genomic hallmarks of ACC progression, evaluate for intratumoral heterogeneity, and assess for pathogenic germline alterations.

The pre-specified hypothesis was that significant differences in the mutational landscape between primary and R/M ACC may help better characterize risk of progression as well as delineate prognosis.

MATERIALS AND METHODS

2. **Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.**

Patients were diagnosed with ACC of varying stages from various institutions (**Table S1**), including patients with recurrent/metastatic disease. Cases were required to have either whole exome sequencing, whole genome sequencing, or targeted panel sequencing.

3. Describe treatments received and how chosen (e.g., randomized or rule-based).

This study was retrospective. Treatments were generally upfront surgery followed by postoperative radiation. Six patients with R/M ACC underwent trials with tyrosine kinase inhibtors based on identified PIK3CA mutations.

4. Describe type of biological material used (including control samples) and methods of preservation and storage.

Tumor specimens for whole exome or whole genome sequencing were obtained at the time of surgery or by biopsy and snap frozen in liquid nitrogen, and stored at -80^{0}C. Primary specimens were obtained prior to treatment, while R/M specimens typically had undergone prior therapy. Blood samples for control specimens were collected by peripheral venous puncture. Tumor specimens for targeted panels were obtained from paraffin embedded tissue, which were stored in room temperature.

5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.

Sequencing data from other institutions were obtained via publicly accessible database, with mutation calls described in their respective publications. All primary ACC cases (n=177) underwent whole exome or whole genome sequencing as described previously. R/M cases either underwent whole exome sequencing (n=16) or targeted sequencing panels (n=851), either by MSK-IMPACT or Foundation Medicine commercial assay. The assays were not performed blinded to the study endpoint.

6. **State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.**

Retrospective case selection was performed based on available studies and unpublished data, spanning 2013 until current time. Stratification by primary vs R/M status was performed.

7. **Precisely define all clinical endpoints examined.**

Overall survival time was defined to be the period from diagnosis (either from primary tumor or R/M tumor) to date of death.

8. **List all candidate variables initially examined or considered for inclusion in models.**

Cox survival analysis was performed based on mutational subgroups. Alterations examined were determined by standard whole genome sequencing, whole exome sequencing, or pre-determined targeted panels (e.g., MSK-IMPACT, Foundation Medicine).

9. **Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.**

Sample size was determined based on available published studies and available unpublished data from participating institutions.

10. **Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.**

For comparing primary vs R/M mutation rates, odds ratios were used for assessing statistical significance. Survival analysis was performed via Kaplan-Meier methodology and compared with the log-rank test. All statistical tests were two-sided, and a p-value <0.05 was considered statistically significant. ACC molecular subgroups were compared for mutual exclusivity using the Benjamini-Hochberg false discovery rate method.

11. **Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.**

Mutations were culled from published datasets, each with its own specific mutation callers and pipeline analysis as previously described. Unpublished datasets from a given institution underwent similar pipelines as published datasets from that respective institution.

RESULTS

12. **Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.**

Collectively, there were 1043 ACC patients studied (177 primary, 868 R/M cases). Of the R/M cases, 94 cases underwent MSK-IMPACT targeted panels, while 730 cases underwent Foundation Medicine

targeted panels. As the Foundation Medicine panels changed over time regarding gene coverage, each case was linked to the particular panel, ensuring correct mutational incidence. All 94 MSK-IMPACT patients had available data to perform survival analysis as well as secondary germline analysis. Of the 94 MSK-IMPACT patients, 58 had available exome and copy number data to assess intratumoral genetic heterogeneity.

13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.

Distribution by anatomic site was 89.8% (head and neck/salivary), 6.8% (lung), and 3.4% (breast) (**Figure S1**). See **Figure 1** for distribution by gender.

14. Show the relation of the marker to standard prognostic variables.

For survival outcomes stratified by mutation or molecular subgroup, see **Figure 3** and **Figure 4**. Significantly poorer prognosis was noted for cases with *NOTCH1* mutations, *NOTCH1* activating mutations, and *KDM6A* mutations, while *MYB(+)/NOTCH1(+)* mutations exhibited the worst outcomes of the molecular subgroups.

15. Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.

See **Figure 3** and **Figure 4**.

16. For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.

No multivariable analysis was performed in this study.

17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.

No estimated effects with confidence intervals were utilized for this study.

18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.

Since most R/M cases were sequenced at higher depth with targeted NGS panels, we assessed the possibility that those mutations enriched in R/M cases might have been mutations with low variant allelic fraction (VAF), below the resolution of WES. None of the mutations that were enriched in R/M cases had VAF<0.05 (a conservative detection threshold in 100x WES11-13) in more than 5% of the cases, with the majority between 0-2% (**Table S5**). To compare the sensitivity of WES (at ~100x) to targeted NGS (at ~600x) for the detection of these enriched mutations, we downsampled the reads from R/M cases

sequenced on the MSK-IMPACT platform to 100x. This minimally altered the resulting VAFs (**Figure S3**), with average change in VAF of 0.011, and only one enriched mutation (1/101, or 1%) was not detected at the downsampled depth (**Table S6**). In addition, a further comparison of primary and R/M ACC cases undergoing sequencing with WES showed clear enrichment of many of the same genes in the original analysis (**Table S7**). Altogether, these analyses confirm that the enriched rate of mutations in these genes in R/M cases is unlikely to be an artifact of differences in sequencing depth.

DISCUSSION

19. Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.

The cohort of over 800 metastatic ACCs is unprecedented for this disease and sizable for any orphan disease. By performing these comparisons in the largest cohort of genomically profiled ACCs to date, new and biologically meaningful findings emerge, such as:

- The highly enriched genes in R/M cases (only *NOTCH1* had been previously reported to be enriched)
- The molecular subgroups of ACC (defined by *MYB*, *NOTCH1*, and *TERT*)
- The patterns of cooperation and mutual exclusivity between genes, including the cooperation between *NOTCH1* and chromatin modifiers, supporting the hypothesis of pioneer and settler factors in the Notch pathway
- The prognostic implications of certain genes
- The analysis of levels of clinical actionability of mutations in ACC (including the PI3K cases, which are the first case series of successful biomarker-driven therapy in ACC)
- The widespread nature of intratumor heterogeneity across a large number of tumors
- The first report of germline mutations in ACC

Limitations include the lack of clinical data for many cases, as well as the different methodologies for sequencing. MYB/MYBL1 status was also not available for all patients. In particular WES/WGS platforms differ from targeted panels, though our Downsampling analysis confirmed that sequencing depth did not seem to impact sensitivity of mutation detection.

20. Discuss implications for future research and clinical value.

In this study, we confirm enrichment of mutations in R/M cases and outline molecular subgroups that may better characterize R/M ACC for prognostic purposes, as well as outline biologic means of progression. The prevalent intratumoral heterogeneity noted also belies the common assumption that ACC harbors a quite genome. The preliminary reporting of pathogenic germline alterations also suggests the unexpected possibility of heredity in this malignancy, though this requires further investigation.