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Keywords

Cutaneous Squamous Cell Carcinoma, CREB-binding protein and Mutation.

Background

Cutaneous squamous cell carcinoma (cSCC) is the second most frequent skin cancer, with an estimated 400,000 new cases reported yearly in the United States (1). Although metastasis is relatively uncommon, prognosis in patients with lymph node positive cSCC is poor and 5-year survival rates are low (2). Furthermore, advanced and often inoperable, loco-regional disease is frequent with cSCC and such patients would benefit from effective, targeted chemotherapies. The hunt for genetic drivers and thus potential targets for cSCC therapy is hampered by a mutation burden greater than any other known cancer with metastatic potential (3-5), only exceeded by basal cell carcinoma of the skin (S1). Deep sequencing of cSCC has identified key driver mutations in tumor suppressor genes (3-5) which are difficult to target therapeutically as it is easier to inhibit the action of a protein than it is to replace its function when missing. A pathway centric approach may be a more appropriate route for therapy development because somatic, cancer-promoting mutations may affect multiple nodes within the same pathway leading to downstream activation (6). We recently identified frequent CARD11 mutations in cSCC, which lead to aberrant NFkB signalling (7). Nuclear factor kappa-b (NFκB) signaling plays a crucial role regulating proliferation and differentiation in the epidermis and alterations can lead to skin pathologies with significant burden to human health such as psoriasis and cSCC. One gene reported as integral to a number of ubiquitous signaling pathways (including NFκB) is *CREBBP* which encodes the protein CBP, a transcriptional co-factor with lysine acetyltransferase activity and close homology to p300 (S2). CREBBP loss of function (LOF) mutations are frequently identified in a range of different cancers such as lymphomas and carcinomas of the bladder and esophagus (S3-5).

Questions Addressed

We previously identified *CREBBP* mutation in 35% of 20 cSCC using exome sequencing (4). Here we address whether *CREBBP* mutation is present in a larger cohort of cSCC samples (n=91).

Experimental Design

Please see supporting information (Data S1).

Results

To define *CREBBP* mutation in a larger cohort of human cSCC we used targeted re-sequencing of 91 samples previously isolated and used to re-sequence potential driver genes such as *NOTCH1*, *TP53* and *CARD11*, as described (4,7). *CREBBP* mutation frequency was high (33/91 cSCC tumors, 36%, compared with 7/20, 35%, in the original exome sequencing cohort). Thus, *CREBBP* is frequently mutated in human cSCC (mutations listed in Supporting Table S1). We next compared the spectrum of *CREBBP* mutation in cSCC with those reported for other tumors in the catalogue of somatic mutations in cancer (COSMIC) (S6) and identified that 50/51 cSCC mutations were missense, in contrast to those reported in COSMIC (n=758) where >25% of mutations were either non-sense or insertion/ deletion mutations leading to frame-shift and a premature termination codon (PTC) (Figure 1A). The majority of such mutations are predicted to result in expression of a truncated protein that is either greatly reduced or absent as a result of non-sense mediated decay (NMD). Analyzing mutation spectrum by tissue type in COSMIC identified certain tumor groups with greater than 33% mutations leading to PTC such as the hematopoetic and lymphatic system as well as esophagus. Bladder harboured 75% non-sense or insertion/ deletion mutations (Figure 1B).

Two previous studies support our observations that loss of function mutation in CREBBP is not prevalent in primary cSCC; Durinck and colleagues identified 1 missense mutation from 8 cSCC (3), while Pickering and colleagues identified 24 mutations in a cohort of 39 aggressive, primary cSCC, 21 of which were missense or silent (5).

Given the spectrum of mutation in cSCC we speculated whether cSCC CREBBP mutation, unlike other tumors with a high CREBBP mutation frequency, does not result in CBP loss of function. To address this immuno-histochemistry (IH) was used to interrogate the expression level and distribution of CREBBP in

normal skin, and cSCC tumors. Using an antibody raised against the C-terminus of CBP nuclear reactivity was clearly demonstrated throughout basal and suprabasal layers of normal skin (Figure 2A) and nuclear reactivity in all 21 cSCC samples tested was also observed. These data are in line with two different antibodies published at The Human Protein Atlas (8) (http://www.proteinatlas.org/ accessed November 17th 2015). In our study, well-differentiated cSCC tumors (n=13) exhibited a pattern of strong positive reactivity but loss in central keratinizing areas (Figure 2B). On the other hand, in 8 cSCC samples with poorly differentiated regions there was greater variability where regions of weak positivity intermixed with negative tumor cells were observed in 3 of 8 high-grade tumours (Figure 2C).

Conclusion

CREBBP is regularly found to be inactivated through loss of function mutations in a range of malignancies (S3-5). In contrast we find cSCC harbor missense mutation, and very few mutations that predict loss of function. Furthermore, we identify CBP in all 21 cSCC examined, indicating that protein is expressed and localizes to the correct compartment. It is unclear at this time, however, what role CBP may have in cSCC, but it is interesting to note that CBP/p300 is critical for efficient removal of UV-induced pyrimidine dimers (S7). Furthermore, recent genomic analysis of 29 lymph node metastases from primary cSCC showed frequent (28%) *CREBBP* mutation (9) but here the majority of alterations (6/8) were truncating or insertion/ deletion mutations predicted to lead to LOF. This result may indicate that CBP inactivation is associated with tumor progression in cSCC and agrees with our IH observation, also in a limited number of samples, that a degree of CBP loss is observed in high grade tumors (Figure 2C). However, these observations will need to be confirmed, and the data presented here together with the recent finding that, unlike *NOTCH1*, *CREBBP* mutation is not frequent or positively selected for in normal, sun-exposed skin (10), suggests that CREBBP mutation is unlikely to be an early event in squamous cell carcinogenesis.

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Author Contributions

A.P.S., C.M.P., I.M.L., C.A.H., K.J.P. and S.A.W. designed the research and obtained funding. C.A.H. and C.M.P. collected samples. A.P.S., K.J.P., S.A.W., A.M., D.X. and J.H.D. performed the experiments. A.P.S., S.A.W., N.d.B., M.D., S.T. and S.T.A. analyzed the data. A.P.S., K.J.P., S.A.W., C.M.P., C.A.H. and I.M.L. wrote the paper.

Conflict of Interest

All authors declare that no conflicts of interest exist with this work.

Supporting Information

Additional supporting data may be found in the supplementary information for this article.

Data S1. Materials and Methods.

Table S1. CREBBP Mutations.

Data S2. Supplementary References.

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Figure Legends

Figure 1: CREBBP mutations in cSCC (n=51) are predominantly missense and distribute evenly with the exception of the bromo and Creb domains as compared to CREBBP mutation in other tumor types (n=785). **A**: CREBBP mutations (indicated by vertical bars) in cSCC identified in this study (**A,** upper cartoon) compared with COSMIC (**A**, lower cartoon), a catalogue of somatic mutations in cancer (accessed June 5th 2015; http://cancer.sanger.ac.uk/cosmic). Fewer nonsense or insertion/ deletion mutations (In/Del) are observed in cSCC (upper cartoon) compared with mutations reported in COSMIC (lower cartoon). Green = missense, Red = nonsense and Yellow = insertion/ deletion. Vertical bars above each CREBBP cartoon indicate two or more mutations present at the same amino acid position; area reflects the number of mutations at a given amino acid residue. Orange horizontal bars below cSCC CREBBP cartoon indicate two regions of low sequence coverage in our study (2-10 reads). Position of protein domains depicted in the CREBBP cartoon is based on NCBI GenPept entry NP_004371, accessed June 15th 2015. **B**: Analysis of COSMIC data identifies varying contribution of nonsense and In/Del mutation in tumors arising at different body sites.

Figure 2: Immunohistochemistry using a CBP (C-1) antibody raised against a C-terminal epitope of the CREBBP protein detected expression in normal skin (**A**, top panel) and comparable or increased levels in well-differentiated cSCC (n=13) (**B**, bottom panel shows higher magnification of top panel). 3/8 poorly differentiated cSCC exhibited regions of weak positivity intermixed with negative cells (**C**, bottom panel shows higher magnification of top panel). Lower panel in **A** shows negative control performed on duplicate tissue sections by omitting the primary antibody incubation step. Bar = 300μm.

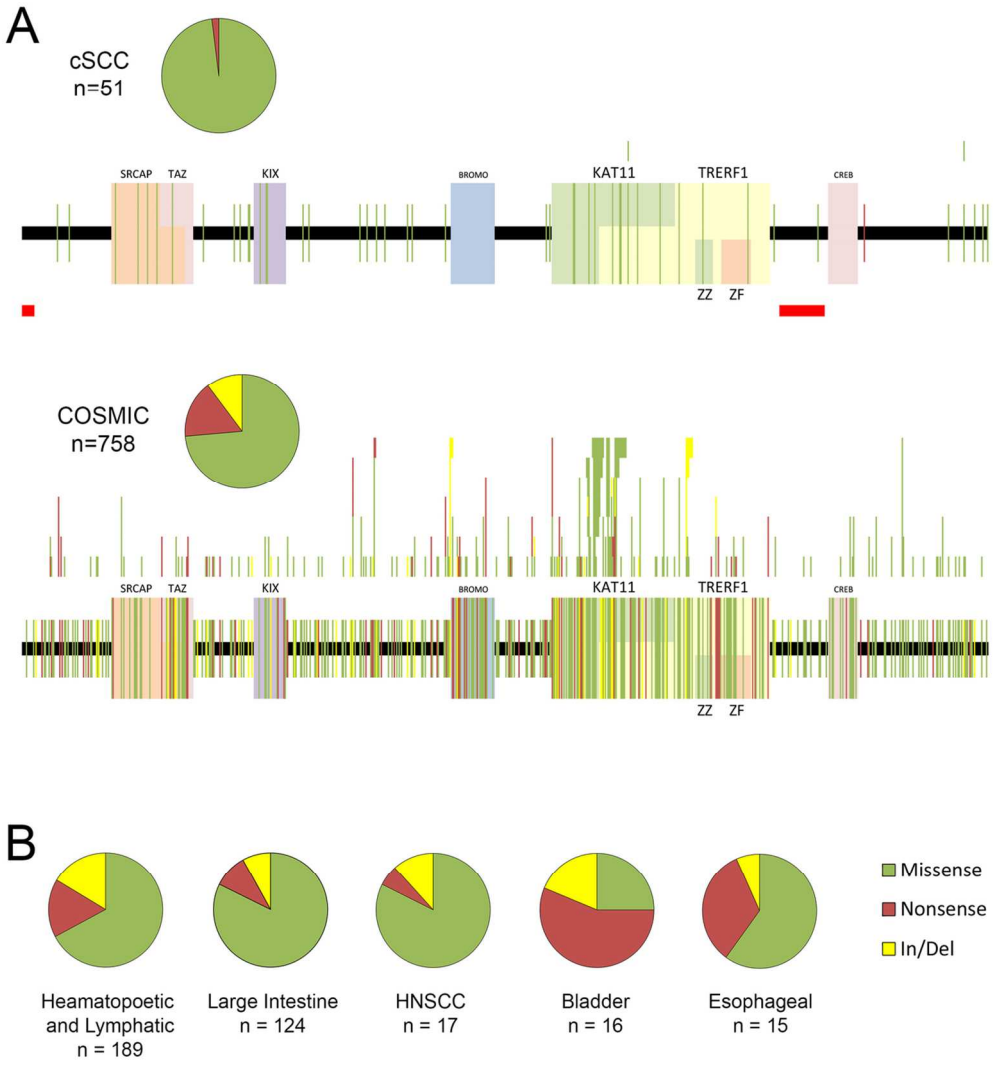

Figure 1
230x247mm (150 x 150 DPI)

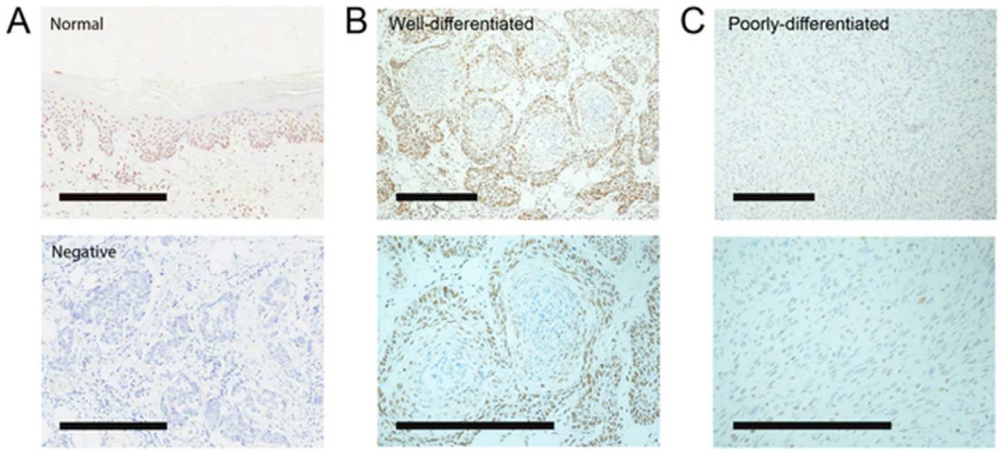

Figure 2
106x48mm (150 x 150 DPI)

Data S1. Materials and Methods

Sequencing

Sample collection and sequencing was carried out as described previously (South et al 2014). Ethical approval for this investigation was obtained from the East London and City Health Authority local ethics committee and the study was conducted according to the Declaration of Helsinki Principles. All patients participating in this study provided written, informed consent. Punch biopsies of cSCC tissue were taken after surgical excision of tumors and immediately snap-frozen in liquid nitrogen with the remainder of the tumor sent for formalin fixation and histopathologic diagnosis. For CREBBP amplification, primers were designed and validated by Fluidigm (Fluidigm Corporation, San Francisco, CA) per recommended guidelines for Roche Titanium sequencing (Roche, Mannheim, Germany). Each primer included sample-specific Fluidigm 454 barcode primer and adapter sequences. Primer sequences are available on request. Reactions contained 50ng genomic DNA, forward and reverse tagged amplification primers (1µM), forward and reverse barcode primers (400nM), 1x Access Array Loading Reagent, 1x FastStart High Fidelity Reaction Buffer, 4.5mM $MgCl_2$, 5% DMSO, 0.05U FastStart High Fidelity Enzyme Blend and PCR-grade nucleotide mix (200µM, Roche). Thermal cycling was performed on the Fluidigm FC1 Cycler per manufacturer guidelines. The resultant libraries were harvested and collected on a microtiter plate and were normalized and pooled before purification using Agencourt AMPure XP system (Beckman, UK) per the manufacturer's protocol. Library components were clonally amplified using the GSJunior emPCR Lib-A Kit (Roche) by inputting 1 molecule of library DNA per capture bead. Pyrosequencing was performed using the GS Junior system (Roche/454 Life Sciences). Mapping and variant calling were performed as described[5]. cSCC mutations were compared to those previously reported in COSMIC[6], an online catalogue of mutations in cancer (http://cancer.sanger.ac.uk/cosmic).

Immunohistochemistry (IH)

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded cSCC tissue specimens and normal skin specimens. Tissue sections were cut at a thickness of 3 μm onto 3-aminopropyltrioxysilane-coated slides and dried overnight in a 45^{o}C oven followed by de-waxing and dehydration in xylene and industrial methylated spirits. Antigen retrieval was performed by incubating sections in TRIS-EDTA-citrate antigen unmasking solution (5g EDTA, 2.5g TRIS base and 3.2g sodium citrate in 1 litre of distilled water, pH adjusted to 8.1 with sodium hydroxide) for 35 minutes in a microwave set to full power. Endogenous peroxidase activity was blocked at ambient temperature in 3% hydrogen peroxide in distilled water for 15 minutes (cSCC) or 45 minutes (normal skin). A monoclonal antibody against the C-terminus of human CREBBP (CBP (C-1): sc-7300; Santa Cruz Biotechnology Inc, Santa Cruz, CA) was applied as primary antibody for 40 minutes at ambient temperature at a dilution of 1:10. Incubation with secondary biotinylated antibody followed by streptavidin-biotin-peroxidase complex was performed using the Vector Elite ABC kit (Vector Laboratories, CA, USA) according to the manufacturer's instructions with diaminobenzidine as the chromogen and haematoxylin as counterstain. Negative control reactions were performed on duplicate tissue sections by omitting the primary antibody incubation step.

Supporting Table S1: 454 *CREBBP* mutations

DNA was isolated from 91 cSCC samples and CREBBP sequencing was carried out using Fluidigm amplicon based 454 pyrosequencing. Mutations meeting filtering criteria are listed

Chr = chromosome, Start and end indicate hg19 position, SNP - variant base, REF = reference base. A, C, G and T indicates nucleotide. TGM denotes moderately differentiated sample, TGP denotes poorly differentiated and TGW d

Mutation identified in 2 Samples

chr	Start	End	SNP	REF	A	C	G	T	No. Quality Reads	No. Variants	%	Sample	aa change	aa pos	REF	Codon change
chr16	3786729	3786730	A	G	6	0	46	0	52	6	12	TGM1	Pro:Leu	1494	C	CCC:CTC
chr16	3830822	3830823	A	G	7	0	58	0	65	7	11	TGM1	Pro:Leu	578	C	CCA:CTA
chr16	3843460	3843461	A	G	10	0	25	0	35	10	29	TGM10	Ser:Leu	381	C	TCG:TTG
chr16	3820826	3820827	A	G	9	0	49	0	58	9	16	TGM12	Pro:Leu	875	C	CCT:CTT
chr16	3786669	3786670	T	C	0	15	0	6	21	6	29	TGM22	Arg:Gln	1514	G	CGG:CAG
chr16	3777819	3777820	A	G	7	0	6	0	13	7	54	TGM23	Pro:Ser	2410	C	CCC:TCC
chr16	3820754	3820755	A	G	16	0	24	0	40	16	40	TGM25	Pro:Leu	899	C	CCC:CTC
chr16	3786663	3786664	G	A	23	0	4	0	27	4	15	TGM28	Ile:Thr	1516	T	ATC:ACC
chr16	3832882	3832883	G	A	33	0	4	0	37	4	11	TGM28	Ser:Pro	459	T	TCT:CCT
chr16	3777899	3777900	A	G	4	0	21	0	25	4	16	TGM3	Pro:Leu	2383	C	CCC:CTC
chr16	3860694	3860695	T	G	0	0	25	4	29	4	14	TGM30	Pro:His	295	C	CCC:CAC
chr16	3777902	3777903	T	G	0	0	24	4	28	4	14	TGM6	Ser:Tyr	2382	C	TCC:TAC
chr16	3843574	3843575	T	C	0	26	0	4	30	4	13	TGM6	Gly:Asp	343	G	GGC:GAC
chr16	3831268	3831269	C	T	0	4	0	30	34	4	12	TGM7	Thr:Ala	538	A	ACA:GCA
chr16	3820698	3820699	T	C	0	33	0	4	37	4	11	TGP10	Val:Ile	918	G	GTC:ATC
chr16	3779536	3779537	G	T	0	0	4	33	37	4	11	TGP13	Lys:Asn	1837	A	AAA:AAC
chr16	3779340	3779341	A	G	7	0	4	0	11	7	64	TGP15	Pro:Ser	1903	C	CCG:TCG
chr16	3819310	3819311	A	G	12	0	20	0	32	12	38	TGP17	Pro:Leu	975	C	CCC:CTC
chr16	3819311	3819312	A	G	12	0	20	0	32	12	38	TGP17	Pro:Ser	975	C	CCC:TCC
chr16	3786167	3786168	C	T	0	4	0	32	36	4	11	TGP3	Ser:Gly	1533	A	AGT:GGT
chr16	3777755	3777756	A	G	4	0	10	0	14	4	29	TGP6	Thr:Met	2431	C	ACG:ATG
chr16	3828780	3828781	T	G	0	0	21	4	25	4	16	TGP6	Leu:Ile	621	C	CTA:ATA
chr16	3781373	3781374	T	C	0	35	0	6	41	6	15	TGW1	Arg:His	1664	G	CGC:CAC
chr16	3790522	3790523	A	T	10	0	0	24	34	10	29	TGW13	His:Leu	1337	A	CAC:CTC
chr16	3789666	3789667	T	C	0	34	0	4	38	4	11	TGW14	Ala:Thr	1398	G	GCT:ACT
chr16	3830831	3830832	T	C	0	38	0	22	60	22	37	TGW14	Ser:Asn	575	G	AGC:AAC
chr16	3830742	3830743	T	C	0	37	0	9	46	9	20	TGW16	Val:Met	605	G	GTG:ATG
chr16	3819274	3819275	C	T	0	6	0	28	34	6	18	TGW18	Gln:Arg	987	A	CAG:CGG
chr16	3824676	3824677	A	G	4	0	10	0	14	4	29	TGW18	Pro:Ser	726	C	CCC:TCC
chr16	3781820	3781821	T	C	0	20	0	4	24	4	17	TGW19	Asp:Asn	1616	G	GAC:AAC
chr16	3900826	3900827	T	C	0	97	0	19	116	19	16	TGW2	Ser:Asn	90	G	AGC:AAC
chr16	3794894	3794895	C	T	0	8	0	17	25	8	32	TGW21	Arg:Gly	1328	A	AGG:GGG
chr16	3828788	3828789	A	G	26	0	0	0	26	26	100	TGW21	Pro:Leu	618	C	CCC:CTC
chr16	3831219	3831220	A	G	7	0	25	0	32	7	22	TGW22	Ser:Phe	554	C	TCC:TTC
chr16	3860623	3860624	T	C	0	24	0	13	37	13	35	TGW22	Val:Ile	319	G	GTC:ATC
chr16	3786091	3786092	C	T	0	8	0	29	37	8	22	TGW27	Gln:Arg	1558	A	CAA:CGA
chr16	3794894	3794895	C	T	1	3	0	12	16	4	25	TGW29	Arg:Gly	1328	A	AGG:GGG
chr16	3777719	3777720	C	T	0	5	0	43	48	5	10	TGW3	Stop:Trp	2443	A	TAG:TGG
chr16	3827637	3827638	T	C	0	45	0	10	55	10	18	TGW3	Val:Met	712	G	GTG:ATG
chr16	3788650	3788651	T	C	0	18	0	4	22	4	18	TGW31	Asp:Asn	1435	G	GAT:AAT
chr16	3820883	3820884	A	G	4	0	25	0	29	4	14	TGW31	Thr:Ile	856	C	ACA:ATA
chr16	3900382	3900383	G	A	43	0	5	0	48	5	10	TGW4	Val:Ala	238	T	GTG:GCG
chr16	3781197	3781198	G	A	32	0	4	0	36	4	11	TGW5	Cys:Arg	1723	T	TGC:CGC
chr16	3786163	3786164	A	G	5	0	16	0	21	5	24	TGW6	Ala:Val	1534	C	GCC:GTC

d below.

denotes well differentiated. Amino acid (aa) change and positon relative to the RefSeq gene NM_004380

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