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**Loss of TGF- $\beta$  drives cSCC from skin stem cells – more evidence**

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Tangible therapeutic advances for aggressive and potentially lethal forms of cutaneous squamous cell carcinoma (cSCC), one of the most common forms of cancer<sup>1</sup>, are currently lacking. As our aging population continues to suffer from its relentless increase in incidence<sup>2</sup>, defining the molecular events that drive cSCC, and importantly the context in which those events occur, clearly represent an urgent research need. Our recent data provides compelling support for the proposed tumour suppressor role of TGF- $\beta$  signalling in skin tumourigenesis, as well as contextual insight into how loss of TGF- $\beta$  signalling, when targeted to specific skin stem cell compartments, permits rapid tumour formation<sup>3</sup>.

In essence, evidence of a key tumour suppressor role for TGF- $\beta$  signalling in human squamo-proliferative disease and invasive cSCC already exists. Convincing reports include the genotype-phenotype correlation between inactivating *TGFBR1* mutation and familial multiple self-healing squamous epithelioma (MSSE)<sup>4</sup>, as well as reports of spontaneous cSCC arising secondary to systemic treatment with the pan-TGF- $\beta$  ligand antibody (GC1008)<sup>5</sup>. In addition, TGF- $\beta$  receptor mutations have also been detected in RAF-kinase induced skin tumours<sup>6</sup>; which is where our study began.

We initially performed targeted deep sequencing of 39 squamo-proliferative lesions from patients treated with vemurafenib (a BRAF inhibitor used to treat advanced melanoma) and identified mutations of both TGF- $\beta$  receptors (*TGFBR1* and *TGFBR2*) in 28% of lesions. Next, using the same targeted sequencing profile, we interrogated 91 sporadic human cSCC and 21 human primary cSCC cell lines and detected mutations of both TGF- $\beta$  receptors in 43% of samples. Crucially, normal blood samples as well as matched normal distant and perilesional skin controls harboured no TGF- $\beta$  receptor mutations, indicating that these mutations appeared to be lesion-specific non-germline events. Consolidating these findings, we detected a similar high frequency of TGF- $\beta$  receptor mutations in a separate cohort of 30 cSCC analysed by whole exome sequencing (WES) – with alterations in a total of 53% of samples analysed using this platform with no mutations in matched normal samples.

Factors such as varying efficiencies in deep sequencing techniques, an exceptionally high mutational burden of cSCC tumours<sup>7</sup> (producing significant background passenger mutation rates) and tumour heterogeneity<sup>7</sup> all contribute to the challenge of confirming driver gene status in cSCC. A robust analytical approach combining computational and statistical models, a functional *in-vitro* analysis of detected mutations and gold-standard *in-vivo* mouse models, not only provided substantial evidence to suggest these mutations were indeed driver mutations, but also the novel finding that *Tgfbr1* deletion appears to drive tumourigenesis when specifically targeted to the bulge stem cell compartment of the hair follicle.

A stringent combination of analytical software programs was used to predict the functional consequence of mutation<sup>3</sup>. This approach predicted that 50% of *TGFBR1* and 70% of detected *TGFBR2* mutations were likely to be functionally damaging. The functional consequence of mutations detected by WES was further assessed by MutsigCV and IntOgen algorithms. Although these algorithms failed to detect *TGFBR1* or *TGFBR2* mutations as significant on an individual basis, it was apparent that this approach failed to account for the potential bias of detecting mutually exclusive mutations that may disrupt signalling pathways. IntOgen analysis also predicts the significance of mutation in signalling pathways and confirmed that the TGF- $\beta$  signalling pathway was significantly mutated. In addition, analysis of variant allelic frequencies (VAF) demonstrated a significant proportion of tumours containing TGF- $\beta$  receptor mutations exhibited the highest VAF's in those mutations compared to VAF's of other common drivers of disease, suggesting they were more likely to be initiating events. In strong supporting this hypothesis, clonal analysis of the WES data using ABSOLUTE predicted that 7 of the 8 TGF- $\beta$  receptor mutations were indeed clonal and therefore likely to represent driver events. Functional evidence out-strips prediction programmes and algorithms. *In-vitro* TGF- $\beta$  reporter gene analysis of a panel of TGF- $\beta$  receptor mutants demonstrated a significant proportion of *TGFBR1* mutants and all *TGFBR2* mutants tested failed to restore active TGF- $\beta$  signalling. In addition, restoring wild-type *TGFBR2* receptor to *TGFBR2*-null cSCC cells restored growth arrest. Taken together, these findings provide convincing evidence that loss of TGF- $\beta$  tumour suppressor function is a common event in cSCC.

Expanding on these findings, we then provide novel gold standard *in-vivo* murine model evidence that the capacity for aberrant TGF- $\beta$  signalling to drive cSCC tumourigenesis, and the kinetics of the tumours that form, are likely dependent on a complex and intriguing cluster of factors, including the affected cellular compartment, the timing of the event, the amplitude of TGF- $\beta$  loss and the co-operative oncogenic driving events. Mirroring the kinetics of vemurafenib-induced tumours, MAPK pathway hyper-activation (through *Braf*<sup>V600E</sup> or *Kras*<sup>G12D</sup> knockin) and TGF- $\beta$  signalling ablation (through *Tgfbr1* deletion) in *Lgr5*<sup>+ve</sup> stem-cells enabled rapid cSCC development, but not when targeted to *Shh*<sup>+ve</sup> hair follicle matrix cells. Mirroring sporadic cSCC, mutation of *Tp53* coupled with *Tgfbr1* deletion in *Lgr5*<sup>+ve</sup> stem-cells also resulted in cSCC development, this time with kinetics akin to sporadic disease. These findings indicate that *Lgr5*<sup>+ve</sup> stem cells may act as cells of origin for cSCC, and that when coupled with MAPK pathway hyper-activation or *Tp53* mutation, within this cellular compartment, loss of TGF- $\beta$  signalling can act as a driving event in skin tumorigenesis.

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