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EGFR and *K-ras* gene mutation status in squamous cell anal carcinoma: a role for concurrent radiation and *EGFR* inhibitors?

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BACKGROUND: There is a growing appreciation for radio-sensitiser use in multi-modal cancer treatment models. Squamous cell anal carcinoma (SCAC) is a rare gastrointestinal tumour traditionally treated with concurrent chemotherapy and radiation. Cetuximab, an epidermal growth factor receptor (*EGFR*) inhibitor, has demonstrated significant efficacy when combined with radiation in squamous cell carcinoma of the head and neck (SccH&N). We wanted to assess *EGFR* and Kirsten-ras (*K-ras*) status in SCAC to see whether it compares with SccH&N.

METHODS: Over 90 SCAC paraffin-embedded biopsies were mounted onto a tissue microarray and were assessed for *EGFR* expression by immunohistochemistry. These samples were also assessed for the most frequently mutated *K-ras* and *EGFR* exons by high-resolution melting analysis.

RESULTS: The *EGFR* was present in over 90% of samples tested. The *K-ras* and *EGFR* mutations were absent in all samples tested, although a synonymous single-nucleotide polymorphism was found in 3 out of 89 samples tested for *EGFR* exon 19.

CONCLUSION: The low rate of *K-ras* and *EGFR* mutations, coupled with the high surface expression of *EGFR*, suggests similarity in the *EGFR* signalling pathway between SCAC and SccH&N, and thus a potential role for *EGFR* inhibitors in SCAC. To our knowledge this is the largest cohort of invasive SCAC samples investigated for *EGFR* and *K-ras* mutations reported to date.

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Squamous cell carcinoma of the anal canal (SCAC) is an epithelial cancer of the alimentary tract that is associated with HPV infection and is radiosensitive (Matczak, 2001). Treatment for the past four decades has involved concurrent fluorouracil/mitomycin and radiation therapy (Glynne-Jones and Mawdsley, 2008). Although effective, treatment is associated with significant morbidity: pancytopenia, severe diarrhoea and dermatitis. Furthermore, patients with progressive disease are left with limited and untested options. Hence, it is important that new treatment strategies be investigated.

In the past two decades a significant amount of research has been spurred by the concept of using tumour-specific biomolecules to increase response rates and decrease morbidity of current cancer treatment regimens (Ciardiello and Tortora, 2008). Furthermore, the more recent discovery that certain biomolecules can sensitise tumours to radiation therapy has opened the door to the field of molecular radiation biology/oncology (Zaidi *et al*, 2009). To date, the most successful example of radiosensitisation by a biomolecule is that of Cetuximab. Recently, the US Food and Drug Administration approved the use of Cetuximab for the treatment of squamous cell carcinoma of the head and neck (SccH&N) in combination with radiation therapy (Ciardiello and Tortora, 2008).

Cetuximab (Erbitux/C225) is an IgG1 chimeric monoclonal antibody against epidermal growth factor receptor (*EGFR*). The *EGFR* (HER-1 or erbB1) is a member of the tyrosine kinase receptor family that also includes HER2 (erbB2), erbB3 and erbB4. It is believed that *EGFR* contributes to tumour development and progression through autocrine stimulation of cell proliferation (Matczak, 2001). The *EGFR* is overexpressed in many common epithelial cancers and it is associated with poor prognosis and treatment response (Nicholson *et al*, 2001; Ang *et al*, 2002).

Several tyrosine kinase activating *EGFR* mutations have been identified, the majority of which are associated with responsiveness to *EGFR* inhibition in non-small cell lung cancer (NSCLC) (Lynch *et al*, 2004). The two most common *EGFR* mutations, representing 85–90% of documented *EGFR* mutations, result from an in-frame deletion of 9–24 nucleotides centred around codons 746–750 in exon 19, or a point mutation at nucleotide 2573 (CTG to CGG) resulting in an arginine for leucine substitution at amino acid 858 (L858R) in exon 21 (Riely *et al*, 2006).

Kirsten-ras (*K-ras*), a small signalling G-protein downstream of *EGFR*, is necessary for *EGFR* signal transduction. Activating

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mutations of the *K-ras* gene have been strongly associated with decreased response to *EGFR* tyrosine kinase inhibitors in NSCLC (Eberhard *et al*, 2005), and decreased response to Cetuximab monotherapy in colorectal cancer (Karapetis *et al*, 2008). The *K-ras* mutations have also been implicated in tumour radioresistance (Bernhard *et al*, 2000). Mutations in codon 12 and 13 of exon 2 account for up to 99% of all *K-ras* mutations (Bos, 1989).

The *EGFR* wild-type expression and gene mutation status, as well as *K-ras* mutation status, has not been well investigated in a large anal carcinoma cohort. This study undertook the task to determine the *EGFR* expression as well as the *K-ras* and *EGFR* gene mutation status, in over 90 anal cancer biopsy samples from the Montreal area.

METHODS

Acquisition of pathology blocks

Following approval from our local ethics board, paraffin-embedded squamous cell anal cancer biopsy and tumour specimens were collected from patients treated in the Montreal area between 1990 and 2010. Written informed consent was obtained from all patients before testing.

DNA extraction

Ninety five tissue-embedded paraffin blocks were cut into 4 μm sections and stained with haematoxylin and eosin (H&E) for tumour cell identification. Six blocks were excluded on pathology re-review because of non-typical features. Thus, 89 samples remained. Paraffin blocks with tumour dense areas were scraped, and DNA was extracted from the scrapings using the MagNA Pure Compact Nucleic Acid Isolation Kit I in the MagNA Pure Compact Instrument (Roche Diagnostics, Burgess Hill, UK), which eluted the purified DNA samples to 50 μl. The concentration and purity of each sample was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples were then diluted to 15 ng μl^{-1} DNA in TE buffer for analysis.

High-resolution DNA melting

The *K-ras* and *EGFR* exons 19 and 21 mutation status was determined using high-resolution melting analysis (HRMA) on PCR-amplified samples. The PCR was performed using Invitrogen HRMA Primers for *K-ras* (Carlsbad, CA, USA), *EGFR* exon 21 and *EGFR* exon 19 on a MJ Research PTC-200 Peltier Thermal cycler (Bio-Rad, Hercules, CA, USA) with 42 cycles ranging in temperature from 65 to 95 °C. Briefly, the reaction mixture for HRMA included 3 μl sterile water, 4 μl LightScanner Master Mix (Idaho Technology, Salt Lake City, UT, USA), 1 μl LC green fluorescent dye, 0.2 μl 1 : 1 mix of forward and reverse primer (25 μM for *EGFR* and 10 μM for *K-ras* assays) and 2 μl of 15 ng μl^{-1} DNA sample.

Samples that resembled the positive controls, or looked like outliers from the negative control, were checked by direct sequencing. A sample was deemed positive if its melting curve resembled the positive control or it landed outside of the wild-type spread.

The PCR for HRMA was performed on a Bio-Rad Hard Shell 96 microplate (Bio-Rad) and all samples were run in duplicate. Positive and negative controls consisted of lung or colorectal cancer samples known to either contain the sought after mutation (*K-ras* codon 12 out of 13 mutation in exon 2, *EGFR* exon 19 in-frame deletion or *EGFR* exon 21 L858R mutation) or known to be wild-type. Two wells containing the PCR mix without DNA were run with each plate to control for contamination. Once PCR was finished, the microplate was loaded into the LightScanner Instrument (HR I 96 Idaho Technology) and the samples were melted and analysed as per the LightScanner Program.

Sample sequencing was done using Applied Biosystems' BigDye ReadyReaction Mix v1.1 (Foster City, CA, USA). Briefly, 2 μl from each sample of interest was diluted in 70 μl of sterile water. Samples were amplified by PCR; the products were elongated using a single primer (forward or reverse) as per the kit protocol. Following the PCR reaction, an AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare, Little Chalfont, UK) was used to remove excess ddNTPs. Samples were dried, 10 μl of HiDi formamide was added to each sample, and then samples were subsequently loaded onto the ABI 3130 Genetic Analyzer (Applied Biosystems).

Tissue microarray and immunohistochemistry

An additional six cases obtained in 2009 and 2010 from the Montreal area were added to those initially obtained for testing with HRMA, and thus 95 tissue-embedded paraffin blocks were cut into 4 μm sections and stained with H&E for tumour cell identification. Next, areas with tumour cells were marked on each H&E slide, and from each case: one core of paraffin-embedded cancer tissue was taken. A tissue microarray (TMA) block consisting of 96 cores was constructed. The TMA was constructed using a Beecher Manual Array (Sun Prairie, WI, USA). Of the initial samples, only 87 had cancerous tissue in the paraffin block following tissue removal for DNA extraction. Eight cases were lost with TMA construction and cutting.

Table 1 details the characteristics of 79 patients whose samples were analysed by immunohistochemistry (IHC). Immunohistochemistry was performed at the Segal Cancer Centre Research Pathology Facility. Four micron formalin-fixed paraffin-embedded sections were cut, placed on SuperFrost/Plus slides (Thermo Fisher Scientific), and dried overnight. Sections were deparaffinised in xylene and rehydrated through graded alcohols to water. Sections were subjected to proteinase K (Sigma-Aldrich, St Louis, MO, USA) antigen retrieval for 5 min at 37 °C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System, Tucson, AZ, USA). All solutions used for automated IHC were from Ventana Medical System. The *EGFR* mab, clone SPM 341 (Catalogue No: 53449), was purchased from AnaSpec (Fremont, CA, USA). Negative control was performed by the omission of the primary antibody. The positive control for wild-type *EGFR* was human placental tissue. Immunostaining for *EGFR* was performed online using a heat protocol.

Sections were scored for staining intensity with conventional light microscopy under $\times 100$ and $\times 400$ magnification by two board-certified anatomic pathologists (RO and AG) blinded to sample identification. The immunostaining was evaluated in two ways: percentage of immunoreactive cells showing membrane staining, and the intensity of that staining graded 1–3 following the description and examples in the DAKO (Agilent Technologies, Santa Clara, CA, USA) monograph for *EGFR* staining in colorectal adenocarinoma. Any membrane staining was considered positive for wild-type *EGFR*, and cases with no membrane staining were scored 0. A semi-quantitative combination score combining staining intensity with the percent of cells

Table 1 Patient characteristics of tested samples

Characteristics	No. of cases for HRMA (89 total)	No. of cases reported for IHC (79 total)
Median age at diagnosis (range)	59.5 (37–88)	59 (37–88)
Sex: male	33 (37%)	29 (37%)
Histologic findings		
Well-differentiated	8 (9%)	9 (11%)
Moderately differentiated	46 (52%)	41 (52%)
Poorly differentiated	18 (20%)	14 (18%)
Baseloid	17 (19%)	15 (19%)

Abbreviations: HRMA = high-resolution melting analysis; IHC = immunohistochemistry.

staining gave a final score: just detectable or weak (1+); moderate (2+); strong/intense (3+).

RESULTS

High-resolution melting analysis

The HRMA is an established technique for accurate, rapid and inexpensive screening of paraffin-embedded tissue samples for *K-ras* and *EGFR* mutations with 100% sensitivity and 90% specificity (Do *et al*, 2008). The technique relies on monitoring the melting curve of a PCR-amplified DNA samples that are saturated with a fluorescent dye. As the temperature rises, the DNA strands dissociate and fluorescent molecules are released. At a characteristic temperature single strands are formed and the fluorescence rapidly drops. The melting curve of a DNA product depends on its GC content, length, sequence and heterogeneity. Mutation scanning in particular is reliant on heteroduplex formation that distorts the shape of the melting curve as compared with a normalised curve of a wild-type reference (Reed *et al*, 2007).

Kirsten-ras exon 2 Zero out of 89 samples screened positive for *K-ras* mutations by HRMA. The results were verified by sequencing the positive control and one sample that was at the upper limit of wild-type spread: sample 68. Sequencing confirmed this sample as negative (Figure 1A).

Epidermal growth factor receptor exon 19 Zero out of 89 samples tested by HRMA screened positive for an exon 19 deletion. One sample, sample 68, was an outlier that neither resembled the wild-type spread, nor the positive control. Sample 68 and the positive control were sequenced. Sample 68 was found to be wild-type, whereas the positive control was confirmed to have an in-frame deletion (Figure 1B).

Epidermal growth factor receptor exon 21 Three out of 89 samples (samples 5, 19, 53) tested positive by HRMA. Subsequently, samples 5, 19 and 53 were sequenced along with the positive control. The three samples (5, 19 and 53) were found to all harbour the same single-nucleotide polymorphism (SNP) in position *rs17290559 (c.2508C>T(p.R836R))* (Figure 1C).

Immunohistochemistry

Epidermal growth factor receptor Ninety-one percent (72 out of 79) of samples stained at least weakly (1+). Forty-nine percent (39 out of 79) stained moderately (2+) or strongly (3+) (Table 2 and Figure 2).

DISCUSSION

Squamous cell anal carcinoma is an uncommon gastrointestinal tumour. To our knowledge, this is the largest cohort of SCAC samples investigated for *EGFR* and *K-ras* mutations reported to date. Although, IHC remains the best choice for routine clinical assessment of *EGFR* status, there is no standardised *EGFR* scoring system, so we chose to use the description and examples in the DAKO monograph for *EGFR* staining in colorectal adenocarinoma (Penault-Llorca *et al*, 2006). Using this scoring system, we found that 91% of our SCAC samples overexpressed *EGFR*, which is within the range of previous reports. In the earliest such study, Hui *et al* analysed 28 anal carcinomas for their immunoreactivity to *EGFR* and found that all but one sample was *EGFR* positive (Hui *et al*, 1999). Lê *et al* (2005) documented universally strong expression of *EGFR* in all 21 tested SCAC, whereas none overexpressed HER 2. Alvarez *et al* (2006) described expression in 55% of 38 SCAC tumour samples examined, among which two thirds had at least moderate staining. The same monoclonal antibodies (*EGFR*, clone 31G7) from the same company were used for all three studies, but they used different antigen retrieval protocols. It is interesting to note that both Alvarez *et al* (2006) and Lê *et al* (2005) observed that tissue storage time has no affect on immunohistochemical detectability of *EGFR* if tissue blocks are used. Zampino *et al* (2009) used an *EGFR* pharmDX assay (DAKO, Agilent Technologies). Of their 12 tested samples, only 7 (58%) stained with the Dako *EGFR* pharmDx Monoclonal Mouse Antibody. More recently, Walker *et al* (2009) found that 96% of 48 invasive SCAC displayed strong membrane immunoreactivity for *EGFR* using clone 3C6 from Ventana. They also tested *in situ* lesions as well as condyloma acuminata, and found that with progressive lesion severity, the

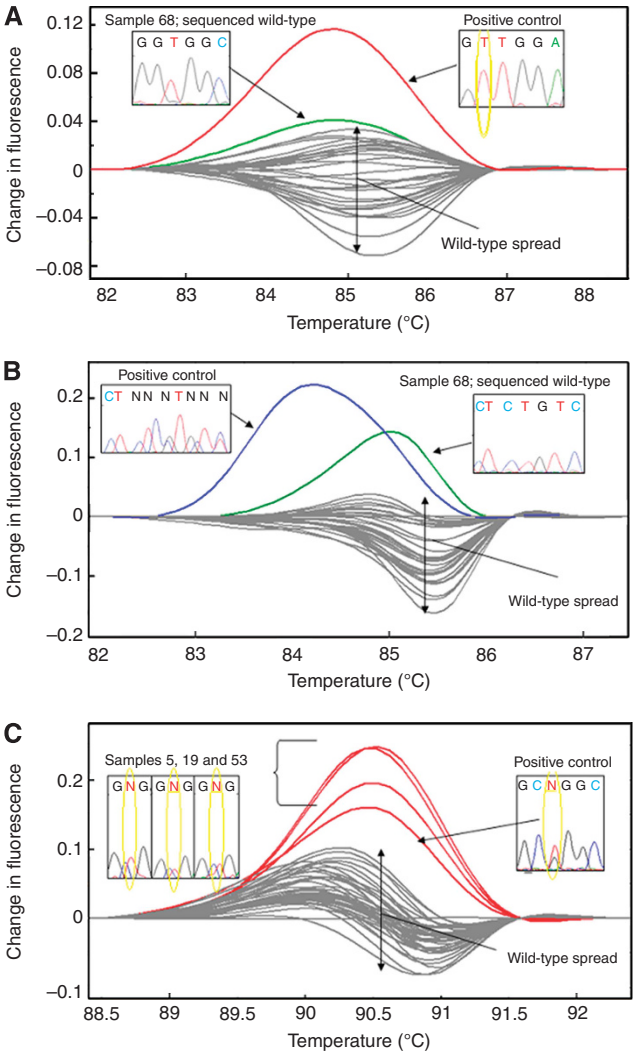

Figure 1 Representative plates from HRMA. Results from one of three 96-well plates done per exon is shown. Samples were run in duplicate, including the positive control, but only one well is shown per sample for graph clarity. The negative control is selected as baseline. Change in fluorescence is calculated by subtracting sample fluorescence from a negative control. (**A**) Kirsten-ras exon 2. The positive control is shown to be mutated in codon 12 out of 13, whereas sample 68 demonstrates the wild-type sequence in this position. (**B**) Epidermal growth factor receptor exon 19. The positive control is shown to have an in-frame deletion at base 112, whereas sample 68 is shown to have the wild-type sequence at this same position. (**C**) Epidermal growth factor receptor exon 21. The positive control is shown to have a CTG to CGG mutation. Samples 5, 19 and 53 are shown to harbour a GTG to GCG single polymorphism at position *rs17290559 (c.2508C>T[p.R836R])*.

Table 2 Assessment of *EGFR* antibody staining intensity

	Number of cases				
Immunoreactivity (%)	**0 (None)**	**1+ (Weak)**	**2+ (Moderate)**	**3+ (Strong)**	**Total**
<20		2	2	1	5
20–40	1[a]	5	5	4	15
41–60	1[a]	14	9	7	31
60–80	1[a]	8	1	7	17
>80	4[a]	4	1	2	11
Total	7	33	18	21	79

Abbreviation: *EGFR* = epidermal growth factor receptor. [a]Staining that was present was cytoplasmic and not membranous, hence counted as absent.

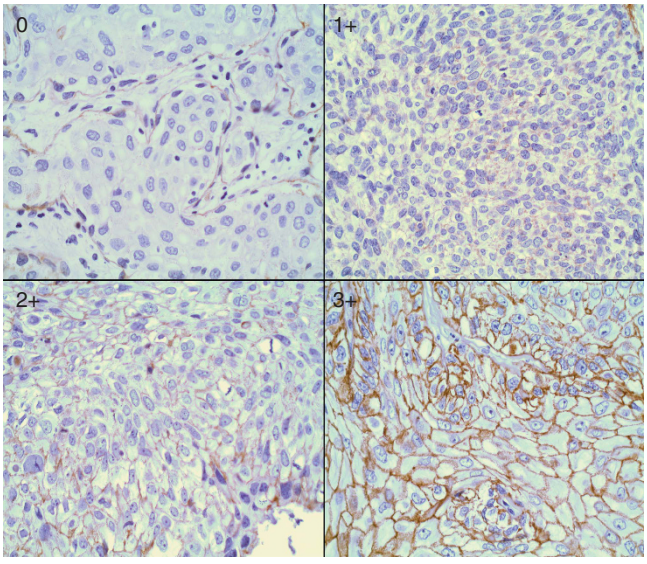

Figure 2 Epidermal growth factor receptor SCAC IHC. Representative photos at × 400 magnification demonstrating 0, 1+, 2+ and 3+ staining, respectively.

percentage of samples staining for *EGFR* increased. Finally, Van Damme *et al* (2010) looked at 43 cases of SCAC. Using clone 31G7 they found that about 84% (36 cases) showed immunoreactivity to *EGFR*, and among these, two thirds exhibited moderate (2+) or strong (3+) staining intensities. Overall, *EGFR* overexpression in SCAC appears to vary from 55 to 100% depending on the immunohistichemical techniques, antigen retrieval methods, antibodies and scoring systems. Our study, using clone SPM 341 from AnaSpec, demonstrated results in keeping with the literature and confirms that patients in the Montreal area have similar *EGFR* expression as those patients previously reported. It is noteworthy that all the samples that failed to show membranous *EGFR* staining did show some intracellular staining, suggesting possible intracellular stores. The significance of this is unknown as it has not been commented on by other authors. Similar to our cohort of samples, the vast majority of SccH&N are also positive for *EGFR* overexpression by IHC (Ang *et al*, 2002; Thariat *et al*, 2012).

No *K-ras* mutations were detected in any of the SCAC samples screened against known positive controls. The *K-ras* mutations appear to be rare in squamous cell tumours as others reported low *K-ras* mutation rates in several epithelial tumours (Bos, 1989). Recently, Van Damme *et al* (2010) sequenced 30 anal cancer samples for *K-ras* mutation, and all samples were wild-type. Similarly, Zampino *et al* (2009) found that their 26 tested samples were wild-type *K-ras* as well.

All tested samples were negative for *EGFR* 19 mutations. One of our samples, sample 68, was an outlier from the wild-type spread, but it was found to harbour no mutation in the amplified exon when sequenced. This sample was likely a true false-positive in both the *K-ras* and *EGFR* exon 19 assays. False positives are well documented in HRMA secondary to degraded DNA from formalin-fixed paraffin-embedded tissue (Do *et al*, 2008). The only other published paper to look at *EGFR* exon 19 mutations in SCAC found no mutations in their cohort of 30 samples (Van Damme *et al*, 2010).

Three samples tested positive by HRMA for *EGFR* exon 21 mutation and were confirmed to have a synonomous SNP by direct sequencing. Both the wild-type and mutated codon code for an arginine. The ability of the HRMA assay to identify the 1-bp change highlights the sensitivity of the technique and further validates its use. Within the limited published literature for *EGFR* mutations in SCAC, Van Damme *et al* (2010) also found no *EGFR* mutations in exon 21 in their 30 investigated samples.

The clinical significance of *K-ras* or *EGFR* mutation with regards to treatment with concurrent radiation and Cetuximab has not been established (Krause and Baumann, 2008). Radiation has been shown to stimulate *EGFR* signalling pathways, and repopulation of epithelial tumour cells after radiation exposure appears to be related to the activation and expression of *EGFR* (Peter *et al*, 1993; Schmidt-Ullrich *et al*, 1997; Petersen *et al*, 2003). Irradiation causes *EGFR* receptor internalisation and transport into the nucleus, binding of the receptor to the catalytic subunit of DNA–protein kinase, and subsequent repair of the lethal double-strand DNA breaks that result from radiation (Dittmann *et al*, 2005). Cetuximab inhibits *EGFR* activation in the absence and presence of radiation (Dittmann *et al*, 2005) and it is hypothesised that by preventing double-strand break repair, cancer cells are deprived of a key mechanism of radioresistance. Thus, when combined with radiotherapy, blockade of *EGFR* signalling could conceivably cause irreparable damage to the cancer cells, and result in cancer cure. Indeed, preliminary clinical trials suggest that effectiveness of radiation does increase if combined with anti-*EGFR* treatment. In a landmark phase III study by Bonner *et al* (2010), Cetuximab combined with radiotherapy *vs* definitive radiotherapy alone at 5 years demonstrated significant benefits in median duration of overall survival (49 *vs* 29.3 months, $P = 0.03$) in patients with SccH&N. Furthermore, there have also been promising results in phase II trials with Cetuximab and concurrent radiation in stage III NSCLC (Jensen *et al*, 2011), and with chemoradiotherapy in locally advanced rectal cancer (Velenik *et al*, 2009). There are already a few reported cases of anal cancer patients being treated by *EGFR* inhibitor monotherapy or in combination with chemotherapy (Saif *et al*, 2011). Overall, there is hope in the field that *EGFR* inhibitors, when combined with current treatment regimens, will help improve survival in refractory anal cancer patients (Lim and Glynne-Jones, 2011).

Presently, there are no clinically established biological markers that can identify patients likely to be radiosensitised by *EGFR* inhibitors, but we do know that SccH&N tumours express *EGFR* and lack both *EGFR* and *K-ras* mutations (Yarbrough *et al*, 1994; Temam *et al*, 2007). Our results confirm that SCAC overexpresses *EGFR* and lacks the most commonly described mutations in *K-ras* and *EGFR*. As SCAC is known to be a highly radiosensitive tumour, we support the introduction of clinical trials testing *EGFR* inhibitors, such as Cetuximab, with concurrent radiotherapy in SCAC.

Conflict of interest

The authors declare no conflict of interest.

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