

EGFR, KRAS, BRAF, and PIK3CA characterization in squamous cell anal cancer

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Summary. Background: Combined chemoradiation therapy is the gold standard in the treatment of squamous cell anal cancer (SCAC). However, even if the response rate is very high, many patients eventually relapse or experience a recurrence, thus requiring an invasive surgical procedure that has severe side effects. Most SCAC tumors overexpress epidermal growth factor receptor (EGFR); therefore, it is reasonable to consider anti-EGFR drugs as a new treatment option, as demonstrated by anecdotal reports. Promising results obtained in other solid tumors, both squamous and non-squamous, have revealed that an increase in the *EGFR* gene copy number may predict the efficacy of anti-EGFR therapies, while the presence of mutations in downstream members of the EGFR pathway may confer resistance. These markers have been only sporadically considered in SCAC.

Methods: We investigated the status of the *EGFR* gene using FISH and examined *KRAS*, *BRAF*, and *PIK3CA* hot-spots mutations using sequencing analysis in a cohort of 84 patients affected by SCAC.

Results: Twenty-eight patients (34%) showed an increase in *EGFR* gene copy number due to amplification (4%) or to polysomy (30%). *KRAS* and *PIK3CA* gene mutations were found in 4 (5%) and 13 patients (16%), respectively. No mutations were found in the *BRAF* gene.

Conclusions: The characterization of the EGFR pathway may help in identifying different subgroups of

SCAC that have specific molecular features, which may have implications in what targeted therapies are used to treat each patient.

Key words: Squamous Cell Anal Cancer, EGFR, KRAS, PIK3CA, EGFR-targeted therapy

Introduction

Squamous cell anal cancer (SCAC) is an uncommon neoplasia, representing 1.5% of all the gastrointestinal malignancies (Martin et al., 2009a). Human papilloma virus (HPV) is considered the major etiologic agent (Frisch, 2002), but many other risk factors have been identified (Palefsky, 1994; Johnson et al., 2004).

Patients with primary SCAC are traditionally treated with chemoradiation, which results in a complete response in up to 90% of cases. In unresponsive or recurrent patients, salvage abdominoperineal resection is recommended (Gervaz et al., 2008; Czito and Willett, 2009; Meyer et al., 2010).

Most SCAC tumors (55-100%) overexpress the epidermal growth factor receptor (EGFR) protein (Le et al., 2005; Alvarez et al., 2006; Walker et al., 2009; Van Damme et al., 2010). EGFR is a tyrosine-kinase transmembrane receptor that regulates cell proliferation and cell survival through the activation of the downstream RAS/RAF/MEK/ERK or PI3K/PTEN/AKT pathways (Wells, 1999).

Recently, EGFR has become increasingly important due to its role as a target in tailored treatments. Various

clinical trials with anti-EGFR drugs have been completed, and many trials are currently ongoing, both for adeno- and squamous cell carcinomas (www.clinicaltrials.gov). In particular, cetuximab, an anti-EGFR monoclonal antibody, has been approved as a therapeutic option for metastatic colorectal cancer (mCRC), as well as head and neck squamous cell cancer (HNSCC) (Gazdar et al., 2010; Kendall et al., 2010; Tejani et al., 2010).

The identification of molecular markers able to predict cetuximab efficacy is crucial for establishing personalized treatment regimens. Therefore, several studies have investigated the role of EGFR and of the members of its downstream pathways responsible for cell signaling. The majority of studies have focused on mCRC treated patients and have reported that the presence of a gain in *EGFR* gene copy number, due to either gene amplification or to chromosome 7 polysomy, can be useful in predicting the patient's response to cetuximab (Cappuzzo et al., 2008; Scartozzi et al., 2009; Personeni et al., 2008). On the contrary, it has been demonstrated that the presence of activating mutations in the downstream elements of the EGFR pathways are able to constitutively activate signal transduction and bypass the cetuximab effect, thereby conferring resistance to this drug (Bertotti et al., 2011; Yonesaka et al., 2011). In particular, it has been shown that mutations in the *KRAS*, *BRAF*, and *PIK3CA* genes may be responsible for cetuximab resistance in mCRC, indicating that these markers are good predictors of therapy inefficacy (Mao et al., 2009; Lin et al., 2011). Surprisingly, the molecular characterization of *EGFR* and of its downstream members has been accomplished only occasionally in HNSCC patients treated with cetuximab, despite the promising value of these factors in predicting the outcome of mCRC (Licitra et al., 2011a; Psyrris et al., 2013; Dorsey et al and Agulnik, 2013).

On the basis of this rationale, cetuximab has also been proposed for the treatment of patients suffering from advanced SCAC, but little is known about the molecular alterations occurring in the *EGFR* gene and in its downstream pathways in this cancer (Phan and Hoff, 2007; Lukan et al., 2009; De Dosso et al., 2010).

We therefore investigated the *EGFR* gene status using fluorescent *in situ* hybridization (FISH), as well as analyzed the *KRAS*, *BRAF*, and *PIK3CA* genes for hot-spot mutations, in order to better understand SCAC pathogenesis and to determine the possible implications of these alterations in patients' management.

Materials and methods

Study population

Eighty-four patients diagnosed with SCAC between 1997 and 2009 were analyzed. Forty-five patients were enrolled at the Institute of Pathology, Locarno, Switzerland, 27 patients were enrolled at the University

School of Medicine, Novara, Italy, and 12 patients were enrolled at the Civil Hospital, Legnano, Milan, Italy. Formalin-fixed paraffin-embedded (FFPE) tissue was available for cytogenetic and molecular analyses in all cases. All histological sections were centrally reviewed by two pathologists (LM and SC), who confirmed the SCAC diagnosis. An HPV test was also performed in all cases. Almost all patients (96%) were found to have an HPV infection. This study was approved by the Institutional Ethical Committee of the Institute of Pathology, Locarno, Switzerland.

Fluorescent *in situ* Hybridization (FISH)

The *EGFR* gene status was analyzed using the dual color FISH assay LSI *EGFR/CEP7* (Abbott Molecular, Baar, Switzerland) on 3- μ m thick FFPE tissue sections, according to the manufacturer's instructions and our previous work (Martin et al., 2012).

FISH handling and interpretation were performed following the European Cytogeneticists Association (ECA) recommendations for FISH on histological sections of solid tumors (<http://www.e-c-a.eu/>). A minimum of 100 morphology-clear, non-overlapping nuclei from at least 8-10 different areas were scored for each patient.

Patients were classified according to the observed abnormalities and the percentage of cells involved. Patients exhibiting one balanced copy of the *EGFR* gene and of the chromosome 7 centromere in >50% of the tumor cells were classified as loss (loss 7), whereas patients with two balanced copies of chromosome 7 in >50% of tumor cells were classified as disomic (D). Patients with 3-4 copies or >4 copies of chromosome 7 in \geq 40% of cells were classified as low polysomic (LP) or high polysomic (HP), respectively, and patients with a ratio (R) of *EGFR* gene/chromosome 7 centromere >2 in \geq 10% of cells were classified as *EGFR* amplified (A).

In addition, the presence of a gain in *EGFR* gene copy number, namely a FISH positive (FISH+) status, was defined for patients carrying \geq 4 copies of the *EGFR* gene in \geq 40% of the cells or for patients with *EGFR* gene amplification. In contrast, patients with \geq 4 copies in <40% of cells were considered FISH negative (FISH-) (Martin et al., 2009b; Varella-Garcia et al., 2009).

Molecular analyses

Genomic DNA was extracted from a single representative FFPE tissue block (containing \geq 70% of neoplastic cells) using the QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Published criteria for accurate block and tumor area selection were applied (Van Krieken et al., 2008). Hot-spot mutations in the *KRAS* (exons 2 and 3), *BRAF* (exon 15), and in the *PIK3CA*, which encoded the p110 α catalytic subunit of PI3K, (exons 9 and 20) genes were investigated using direct sequencing. The PCR conditions were previously reported (Frattini et al., 2004,

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2007; Di Nicolantonio et al., 2008; Sartore-Bianchi et al., 2009). All samples were subjected to automated sequencing using an ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA), and the data were analyzed using the Sequencing Navigator Software (Applied Biosystems). All mutated cases were confirmed by at least two independent PCR reactions.

Statistical analysis

A chi-square test was conducted to assess the association between the clinical-pathological data and the molecular data. Fisher's exact test was used when expected counts were <5 . The level of significance was set at $p=0.05$.

Results

Patients

The investigated cohort of SCAC patients included 63 (75%) women and 21 (25%) men. The age at the time of diagnosis ranged from 41 to 95 years, and 7 patients (8%) were younger than 50.

The majority of the tumors were moderately to poorly differentiated, but all cases were infiltrating tumors.

EGFR FISH

FISH analysis was successful in 82 cases (98%) and failed in 2 cases due to poor hybridization signals. *EGFR*

gene amplification was observed in 3 patients (4%). Of these, 2 patients presented a high level of amplification ($R>10$), with large clusters of signals in all the cells (Fig. 1a), while the last patient showed a low level of amplification ($2<R<3$) at different cellular foci corresponding to 50% of cells of the entire section. HP was found in 13 patients (16%), LP was found in 28 (34%) patients, D was found in 36 (44%) patients (Fig. 1b), and the loss of chromosome 7 was observed in 2 (2%) patients.

Overall, 28 patients (34%) were considered to be *EGFR* FISH+, and 54 patients (66%) were *EGFR* FISH- (Table 1).

KRAS, BRAF, and PIK3CA sequencing

Mutations in the *KRAS* gene were found in 4 out of the 82 (5%) analyzed cases. All mutations were classical, high frequency alterations, including the G12D change (GGTgAT, GlyAsp) in 3 cases (for an example, see Fig. 2a) and the G12V mutation (GGT→GtT, Gly→Val) in the remaining case.

Mutations in the *PIK3CA* gene were found in 13 out of the 79 analyzable cases (16%). Ten mutations occurred in exon 9, and 3 mutations occurred in exon 20. Mutations in exon 9 involved codon 545 in 7 cases, codon 546 in 2 cases, and codon 542 in 1 case. At codon 545, all the mutations corresponded to the transition G→A in the first base of the codon (GAG→aAG, Glu→Lys, E545K; Fig. 2b). Mutations in codon 546 also involved the first base, with a C→A substitution in 1 case (CAG→aAG, Gln→Lys, Q546K) and a C→G

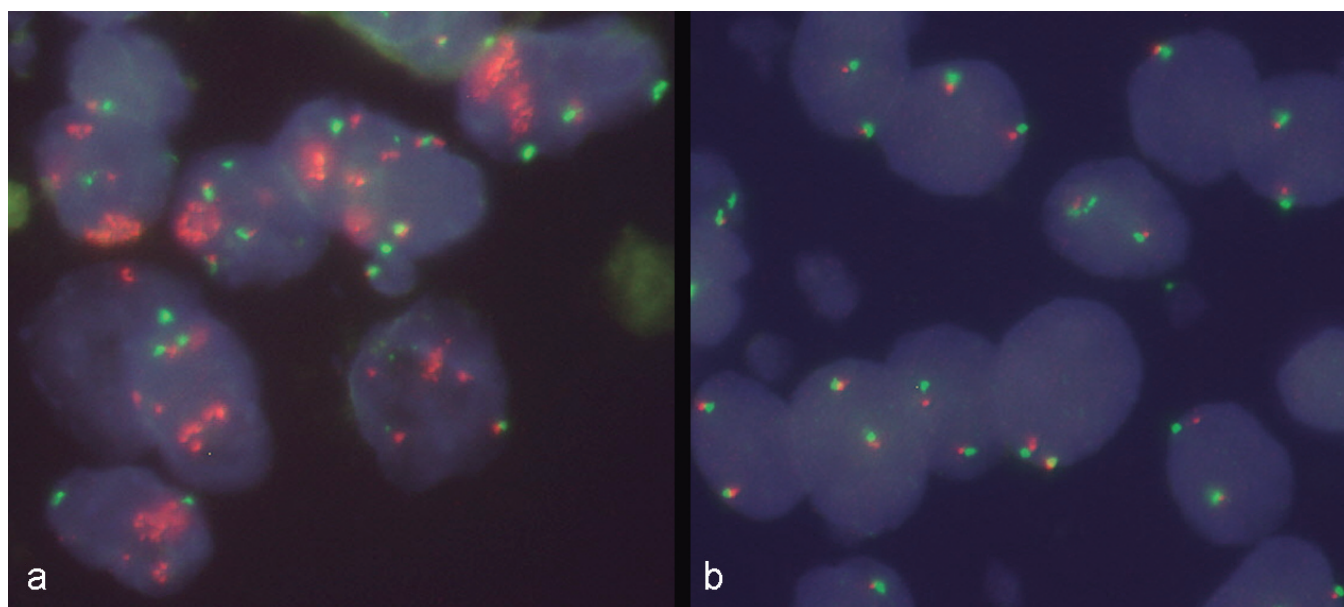


Fig. 1. *EGFR* FISH assay on FFPE tissue section from SCAC patients. **a.** FISH+ case showing *EGFR* gene amplification in clustered signals (red signals). **b.** FISH- case showing two copies of the *EGFR* gene and disomy of chromosome 7 (green signals).

substitution in another case (CAG→gAG, Gln→Glu, Q546E). In codon 542, the mutation was present in the first base (GAA→aAA Glu→Lys, E542K). In exon 20, 2 mutations occurred at the classical codon 1047 (CAT→CgT, His→Arg, H1047R), and the other mutation occurred at codon 1048 (CAT→tAT, His→Tyr, H1048Y).

No mutations were found in exon 15 of the *BRAF* gene (Table 1).

Overall, downstream members of the EGFR pathway were altered in 17 patients (20%).

No patients exhibited concomitant mutations in the *KRAS* and *PIK3CA* genes.

Deregulation of EGFR and its downstream pathways

Out of the 28 *EGFR* FISH+ patients, 3 patients showed a concomitant mutation in the *KRAS* gene, and 3

Table 1. Cytogenetic and molecular features of the investigated SCAC patients.

Gene	Status	N	%
<i>EGFR</i> (N: 82, n.e: 2)	L	2	2%
	D	36	44%
	LP	28	34%
	HP	13	16%
	A	3	4%
	FISH -	54	66%
	FISH+	28	34%
<i>KRAS</i> (N: 82, n.e: 2)	Wt	78	95%
	Mut	4	5%
<i>PIK3CA</i> (N: 79, n.e: 5)	Wt	66	83%
	Mut ex. 9	10	13%
	Mut ex. 20	3	4%
<i>BRAF</i> (N: 80, n.e: 4)	Wt	80	100%
	Mut	0	0%

A: amplification; D: disomy; ex: exon; FISH+: patients carrying ≥ 4 copies of *EGFR* in $\geq 40\%$ of cells or *EGFR* gene amplification; FISH-: patients with ≥ 4 copies in $<40\%$ of cells; HP: high polysomy; L: loss; LP: low polysomy; Mut: presence of a mutation; N: number of evaluable cases; n.e.: not evaluable cases; Wt: wild-type.

patients showed a concomitant mutation in the *PIK3CA* exon 9. Overall, 22 patients (26%) displayed a gain in *EGFR* gene copy number as the sole abnormality.

Molecular alterations and clinical-pathological features

No correlations were found among the alterations in the investigated genes (*EGFR*, *KRAS*, and *PIK3CA*) and the clinical pathological features, such as age, sex, tumor grade, infiltration, differentiation, ulceration, keratinization, and HPV infection.

Discussion

This study concurrently characterized the deregulation of *EGFR* and of its downstream members, *KRAS*, *BRAF*, and *PIK3CA*, in a large cohort of SCAC patients. In our series, one third of the patients (34%) showed an *EGFR* FISH+ profile. The increase in *EGFR* gene copy number was mainly due to polysomy (30%) rather than gene amplification (4%), confirming the few published studies that reported a polysomy rate of 9-33% in SCAC (Le et al., 2005; Alvarez et al., 2006; Walker et al., 2009; Van Damme et al., 2010). These *EGFR* FISH+ results are in line with recent observations in squamous cell cancers occurring at other sites, such as an *EGFR* FISH+ profile in 10% of esophageal squamous cell carcinomas, in 8-36% of oral squamous cell carcinomas, and in 27% of lung squamous cell carcinomas (Nakata et al., 2011; Lee et al., 2012; Yang et al., 2012; Bernardes et al., 2013; Rössle et al., 2013). These demonstrations suggest that polysomy of chromosome 7 might be a common event in the carcinogenesis of tumors with this histology. Interestingly, we found *EGFR* gene amplification in 3 patients. However, the pattern of gene amplification was not the same among these cases. Two patients showed a homogeneous high level of amplification in almost all the entire sample, whereas one patient showed a low level of amplification in only a fraction of the neoplastic cells. These observations suggest that different biological mechanisms might result in *EGFR* gene amplification, as observed in other

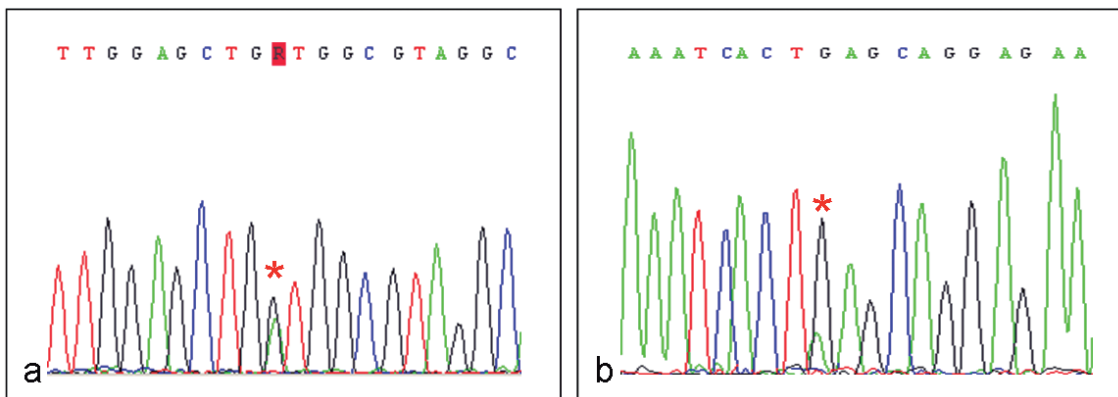


Fig. 2. Mutational profiles of SCAC patients. **a.** *KRAS* G12D change (GGT→GaT, Gly→Asp). **b.** *PIK3CA* exon 9 mutation in codon 545 (GAG→aAG, Glu→Lys, E545K).

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solid tumors. Of note, *EGFR* gene amplification has never been observed in SCAC before (Le et al., 2005; Alvarez et al., 2006; Walker et al., 2009; Van Damme et al., 2010), but it has been described in squamous cell carcinomas of head and neck (11%), tonsil (16%), and esophagus (7%-21%) (Van Damme et al., 2010; Licitra et al., 2011b; Yang et al., 2012; Kato et al., 2013).

Several studies on cetuximab have demonstrated that mCRC patients that have a gain in *EGFR* gene copy number have better response rates and improved survival than FISH- patients; this suggests that *EGFR* FISH+ status is a predictive marker for the efficacy of anti-EGFR therapy (Frattini et al., 2007; Cappuzzo et al., 2008; Scartozzi et al., 2009; Personeni et al., 2008). However, this association has not been confirmed in *EGFR* FISH+ HNSCC patients treated with cetuximab (Licitra et al., 2011b). At the moment, concerns about *EGFR* FISH interpretation and the lack of a standardized cut-off have limited the use of this test in clinical practice (Martin et al., 2009a,b; Sartore-Bianchi et al., 2012). Most likely, further prospective studies will establish the predictive usefulness of *EGFR* gene status in cetuximab management of squamous cell cancers, including SCAC.

In regards to EGFR downstream pathway members, we found *KRAS* gene mutations in 4 patients (5%). These mutations occurred in exon 2 of the *KRAS* gene and were represented by the G12D and G12V changes. Activating mutations in *KRAS* are mostly found in a hot-spot region comprised of codons 12 and 13 of exon 2. All these changes have the same effect on the conformational status of Ras GAP GTPase protein, leading to a constitutively active form that is unable to release GDP (Ellis and Clark, 2000). The alterations found in our cohort are the same as ones reported in colorectal, pancreatic, lung, and biliary tract cancers (<http://cancer.sanger.ac.uk/cosmic>). These results are in line with the published studies concerning SCAC, where *KRAS* mutations have been found only sporadically (Lukan et al., 2009; Zampino et al., 2009; Van Damme et al., 2010; Paliga et al., 2012). These data are also in line with general squamous cell histology, as *KRAS* mutations are infrequent events in squamous cell carcinoma of the penis, tonsil, lung, esophagus, and head and neck (Van Damme et al., 2010; Gonzaga et al., 2012; De Carvalho et al., 2013; Fiala et al., 2013; Gou et al., 2013).

In addition to the *KRAS* gene, *BRAF* is another relevant gene downstream of EGFR. We searched for the presence of the most common type of mutation (V600E), which leads to a 500-fold increase in the *in vitro* kinase activity, thus inducing cell proliferation and transformation (Wan et al., 2004). We did not find any mutations in the *BRAF* gene; this result confirms many studies on other types of squamous cell cancers that did not report any *BRAF* mutation, even in large series (Gonzaga et al., 2012; De Carvalho et al., 2013; Gou et al., 2013; Shigaki et al., 2013). However, there is a

report of a single patient affected by squamous cell carcinoma of the lung with a *BRAF* mutation (Alrifai et al., 2013). Altogether, these results indicate that *BRAF* plays a minor role, if any, in SCAC; this is in contrast to what has been reported for non-squamous cell carcinomas, such as thyroid cancers, melanomas, and in particular CRC (Bardelli and Siena, 2010; Mao et al., 2011; Muhammad et al., 2013).

In this study we also analyzed the *PIK3CA* gene, focusing our attention on exons 9 and 20. These two conserved regions, which encode the helical and the kinase domains of the protein, respectively, contain the major and most frequent *PIK3CA* mutations, such as E545K, E542K, and H1047R. All of these mutations are non-synonymous missense mutations that confer a constitutive kinase activity, resulting in cell proliferation (Samuels et al., 2005). In our cohort, we identified 13 patients (16%) with alterations in the *PIK3CA* gene. The majority of these patients (10) exhibited mutations in exon 9, while 3 cases were mutated in exon 20. At the moment, there is only one study regarding *PIK3CA* mutations in SCAC. This study observed a 4% mutation rate, with more frequent mutations in exon 20 (Patel et al., 2007). The variability in the frequencies of *PIK3CA* mutations is similar to those observed in squamous cell carcinomas occurring in other sites, such as lung, esophagus, head and neck, oral cavity, and orofaringe; these cancers demonstrate mutation rates ranging from 4 to 28%, with the exception of one study on 94 HNSCC where no mutations were observed in this gene (Kozaki et al., 2006; Mori et al., 2008; Murugan et al., 2008; Akagi et al., 2009; Cohen et al., 2011; Maeng et al., 2012; De Carvalho et al., 2013; Fiala et al., 2013; Nichols et al., 2013). The *PIK3CA* mutation frequency in SCAC and in other squamous cell cancers seems to be slightly lower than those reported in other solid tumors such as breast (27%), endometrial (23%), colorectal (15%), urinary tract (17%), and ovarian (8%) cancers (<http://cancer.sanger.ac.uk/cosmic>).

The molecular characterization of *EGFR* and of its downstream pathway members may provide useful information in the selection of targeted therapies for patients. In light of what has been approved to treat cancers with similar histology, such as HNSCC, cetuximab has also been proposed for the treatment of patients suffering from advanced SCAC (Le et al., 2005; Alvarez et al., 2006; Walker et al., 2009; Van Damme et al., 2010).

A few case reports have described cetuximab activity in SCAC. Phan and colleagues observed a positive response to the combination of cetuximab and irinotecan in a patient with irinotecan-refractory SCAC (Phan and Hoff, 2007). Similarly, we reported the efficacy of cetuximab in combination with irinotecan in a patient with refractory SCAC; this patient exhibited a slight increase in *EGFR* gene copy number but had a wild-type *KRAS* gene (De Dosso et al., 2010). Lukan and collaborators observed a disease control in 5 out of 7

metastatic SCAC patients treated with cetuximab. All 5 of the responsive patients carried a wild-type *KRAS* gene, whereas both patients who did not respond had a *KRAS* gene mutation (Lukan et al., 2009). Very recently, Bermettler reported the efficacy of cetuximab in combination with FOLFIRI in a wild-type *KRAS* metastatic SCAC (Barmettler et al., 2012).

These anecdotal findings underscore similar well known findings observed in mCRC and HNSCC (Lievre et al., 2006; Karapetis et al., 2008; Mao et al., 2009; Van Cutsem et al., 2009; Bardelli and Siena, 2010; De Roock et al., 2010; Smilek et al., 2012; Okano et al., 2013) and reinforce the notion that *KRAS* mutations may be a crucial factor in determining a patient's resistance to cetuximab, regardless of the histological subtype. The same concept has been proposed for *PIK3CA* gene mutations; however, at the moment, information about its predictive role is still contradictory in mCRC patients and is completely lacking in squamous cell carcinomas, including SCAC (Bardelli and Siena, 2010; Mao et al., 2012). Further studies are needed to examine these issues.

It is well known that SCAC is characterized by a very high frequency of HPV infection, typically due to the presence of the HPV 16 subtype (Frisch, 2002). Our data indicated that 96% of our patients were infected with HPV, and these findings are in line with those reported in the literature. In other HPV-related neoplastic diseases, such as HNSCC and penile carcinomas (Stankiewicz et al., 2011; Troy et al., 2013), as well as *in vitro* cervical cancer cell lines and *in vivo* xenografts (Deberne et al., 2013), a correlation has been proposed between the presence of the HPV genome and EGFR deregulation, as evaluated by protein expression (Walker et al., 2009). A very recent study in HPV positive HNSCC patients investigated the relationship between *PIK3CA* activation *via* mutation and the presence of HPV infection (Nichols et al., 2013) and found that this association was a frequent event. However, since we observed HPV infection in nearly all our cases, we could not speculate about the link between HPV infection and the EGFR pathways in SCAC.

In conclusion, we performed a molecular characterization of the *EGFR*, *KRAS*, *BRAF* and *PIK3CA* genes in a large cohort of SCAC patients and found that: i) an *EGFR* FISH+ profile occurs in one third of the patients; ii) *EGFR* gene amplification is a rare, but not absent, event in SCAC; iii) *KRAS* and *PIK3CA* mutations may occur in a subset of SCAC patients; and iv) *BRAF* gene mutations are lacking or at least are very rare in SCAC.

These molecular alterations could help to identify different subgroups of SCAC patients and may have potential implications on a patient's treatment options, including drugs that directly target *EGFR*, which are currently under evaluation, or those directed against *KRAS* and *PIK3CA* (Psyrris et al., 2013), which have been recently developed (Rebucci et al., 2011).

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Competing interest. No conflicts of interest are declared.

Authors's contributions. All authors participated in study conception, drafting, and approval of the manuscript.

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