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# EXTENSIVE CHARACTERIZATION OF EGFR AND OF ITS DOWNSTREAM PATHWAYS MAY HELP INTEGRATING THE USE OF EGFR-TARGETED THERAPIES IN PATIENTS WITH SQUAMOUS CELL ANAL CANCER

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# ABSTRACT

*Background.* Squamous cell anal cancer (SCAC) is a rare disease, representing 1.5% of all the gastrointestinal tumors. Patients with SCAC are traditionally managed with chemoradiation therapy. When non-response or recurrence occur, abdominoperitoneal resection of the anal canal is recommended. New therapeutic options are wondered to overcome the severe side effects of this surgical procedure.

It has been demonstrated that the majority of SCAC (55-100%) are characterized by EGFR (Epidermal Growth Factor Receptor) deregulation.

Recently, the anti-EGFR monoclonal antibody cetuximab has been FDA and EMA approved for the treatment of head and neck squamous cell cancer and metastatic colorectal cancer (mCRC). Only sporadic studies investigated the use of cetuximab in SCAC, reporting activity in very few patients.

To date, in SCAC little is known about EGFR and EGFR-downstream members alterations, which are well characterized predictive markers of anti-EGFR therapies efficacy (i.e.: EGFR gene copy number gain) or impairment (KRAS, BRAF and PIK3CA mutations) in patients affected by mCRC.

The aim of this study was to extensively characterize EGFR, KRAS, BRAF and PIK3CA alterations in a series of patients with SCAC.

*Patients and Methods.* We centrally investigated a cohort of 93 SCAC patients diagnosed between 1997 and 2010. Formalin-fixed paraffin-embedded tissue samples were collected from the Departments of Pathology in Locarno, Legnano, Novara and Modena. EGFR gene copy number was

evaluated by fluorescence in situ hybridization (FISH) using the LSI EGFR/CEP7 dual colour assay. HPV detection was performed using the INNO-LiPA HPV genotyping extra amp kit. Hot-spot mutations in KRAS (exons 2 and 3), BRAF (exon 15) and PIK3CA (exons 9 and 20) genes were investigated by direct sequencing.

*Results.* EGFR gene copy number gain (FISH+) was found in 33/90 (37%) evaluable patients. HPV infection was revealed in 87/91 (96%) patients; the majority of them (79/87=91%) carried high-risk HPV types. KRAS gene mutations were found in 4/91 (4%) analyzable patients. BRAF gene was always wild-type (wt). PIK3CA gene mutations were found in 13/89 (15%) analyzable cases (10 mutations occurred in exon 9 and 3 in exon 20). No patient showed concomitant mutations in KRAS and PIK3CA genes. Among 33 FISH+ cases, 3 (10%) patients showed a mutation in KRAS gene and 3 (10%) patients showed a mutation in PIK3CA exon 9.

*Conclusion.* In addition to EGFR gene deregulation, KRAS and PIK3CA mutations are involved in SCAC carcinogenesis. Considering studies on mCRC demonstrating that a EGFR FISH+/ KRAS wt/PIK3CA wt or exon 9 mutated status is associated with clinical benefit from cetuximab, it can be hypothesized that a subgroup of patients affected by SCAC (approximately 33%) might have a proficient molecular profile with respect to anti-EGFR treatments. Our results, therefore, suggest a possible integration of EGFR-targeted therapies in SCAC and emphasize the need of molecular analyses for a better patients' selection.

# ABBREVIATIONS

| | |
|---|---|
| **5-FU** | 5-Fluorouracil |
| **A** | Amplification |
| **APR** | Abdomino-Perineal Resection |
| **CEP7** | Chromosome Enumeration Probe 7 |
| **CR** | Complete Response |
| **CRC** | ColoRectal Cancer |
| **D** | Disomic |
| **DFS** | Disease Free Survival |
| **EBRT** | External Beam Radiation Therapy |
| **EGFR** | Epidermal Growth Factor Receptor |
| **EMA** | European Medicines Agency |
| **FDA** | Food and Drug Administration |
| **FFPE** | Formalin-Fixed Paraffin-Embedded |
| **FISH** | Fluorescence In Situ Hybridization |
| **FISH+** | FISH positive |
| **FISH-** | FISH negative |
| **G** | Grade |
| **HER** | Human Epidermal Receptor |
| **HIV** | Human Immunodeficiency Virus |
| **HNSCC** | Head and Neck Squamous Cell Carcinoma |
| **HP** | High Poysomy |
| **HPV** | Human Papilloma Virus |
| **HPV+** | HPV positive |
| **HPV-** | HPV negative |
| **HR** | High Risk HPV type |
| **IHC** | ImmunoHistoChemistry |
| **IV** | Intravenous |
| **LP** | Low Polysomy |
| **LR** | Low Risk HPV type |
| **MCC** | Mitomycin C |
| **mCRC** | metastatic ColoRectal Cancer |

| | |
|---|---|
| **MoAb** | Monoclonal Antibody |
| **NSCLC** | Non-Small-Cell Lung Cancer |
| **OS** | Overall Survival |
| **PCR** | Polymerase Chain Reaction |
| **PI3K** | PhosphoInositide 3-Kinase |
| **QOL** | Quality Of Life |
| **R** | Ratio (EGFR gene vs Centromere of chromosome 7) |
| **RT** | Radiation Therapy |
| **SCAC** | Squamous Cell Anal Cancer |
| **SCC** | Squamous Cell Carcinoma |
| **TK** | Tyrosine Kinase |
| **TKI** | Tyrosine Kinase Inhibitor |
| **TNM** | Tumor–Node–Metastasis staging system |
| **WT** | Wild-Type |

# 1 BACKGROUND

Anal canal carcinoma is a relatively rare disease, and its most common histological type is squamous cell carcinoma (SCC) (Martin FT. et al, 2009). Human papilloma virus (HPV) is considered the major etiologic agent, but many other different risk factors have been identified (Frisch M., 2002).

Despite the increasing numbers of patients with anal cancer, little has changed in the paradigm for the treatment and outcome of this disease (Uronis HE. et al, 2007). Patients with primary squamous cell anal cancer (SCAC) are traditionally managed with chemoradiation, which results in complete response (CR) in up to 90% of cases. In non-responders or recurrent patients, salvage abdomino-perineal resection (APR) is recommended (Gervaz P. et al, 2008; Czito BG. et al, 2009; Meyer J. et al, 2010).

## 1.1 EPIDEMIOLOGY AND RISK FACTORS

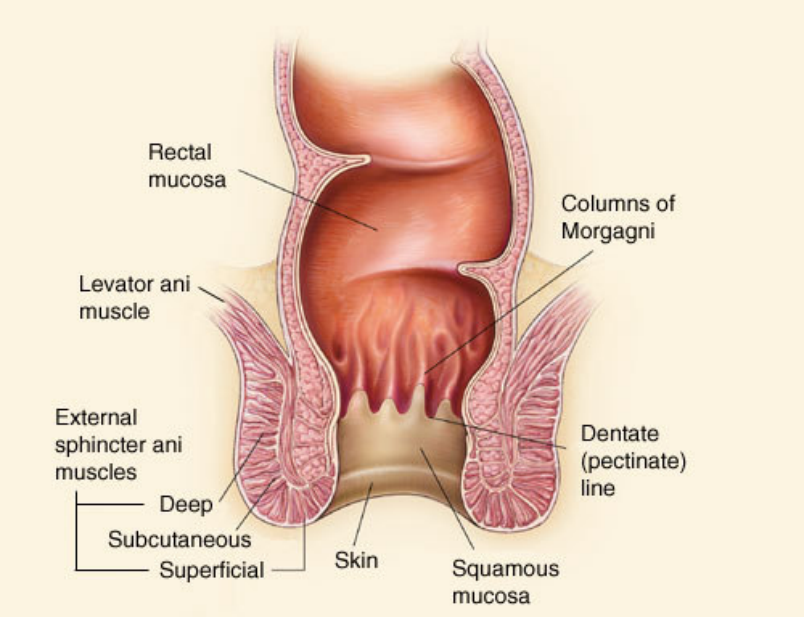
SCAC is a rare tumor and accounts for only 1.5% of cases of gastrointestinal tract cancer (Martin FT. et al, 2009). The age-adjusted incidence rate is 1.5 per 100,000 both in Ticino and in Italy. Of note, the incidence of anal cancer is much higher in men who practice anoreceptive intercourse and in those with human immunodeficiency virus (HIV). Historically, anal cancer was believed to develop as a result of chronic irritation resulting from benign conditions, including hemorrhoids and fissures, and there was also thought to be an association with inflammatory bowel disease (Frisch M., 2002). Several studies over the last decade have found that this is not the case, but have

identified other risk factors, including a history of persistent high-risk genotype HPV infection, infection with multiple HPV genotypes, cervical dysplasia or cancer, HIV seropositivity, low CD4 count, cigarette smoking, anoreceptive intercourse, and immunosuppression following solid organ transplant (Palefsky JM., 1994; Johnson LG. et al, 2004).

## 1.2 ANATOMY AND HISTOLOGY

The anal canal is defined as the terminal part of the large intestine, beginning at the upper surface of the anorectal ring and passing through the pelvic floor to end at the anus. The most important macroscopic landmark in the mucosa is the dentate (pectinate) line composed of the anal valves and the bases of the anal columns (Figure 1) (Ryan DP. et al, 2000).

**Figure 1**




Anatomy of anal canal (source: www.medscape.com)

Histologically, the mucosa can be divided into three zones. The upper part is covered with colorectal type mucosa. The middle part is the anal transitional zone, which is covered by a specialized epithelium with varying appearances; it extends from the dentate line and on average 0.5-1.0 cm upwards. The lower part extends from the dentate line and downwards to the anal verge and has formerly been called the pecten. It is covered by squamous epithelium, which may be partly keratinized, particularly in case of mucosal prolapse. The perianal skin (the anal margin) is defined by the appearance of pigmented skin appendages, immediately surrounding the anal orifice, extending laterally to a radius of about 5 cm. There exists no generally accepted definition of its outer limit. The term anus refers to the distal external aperture of the alimentary tract (Tanum G., 1992).

The lymphatic drainage varies in different parts of the canal in relation to the dentate line. Proximally drainage is to perirectal nodes along the inferior mesenteric artery. Lymph from immediately above the dentate line drains to internal pudendal nodes and to the internal iliac system. Infra-dentate and perianal skin drains to the inguinal, femoral and external iliac nodes (Ryan DP. et al, 2000).

Despite its short length, the anal canal produces a variety of tumor types reflecting its complex anatomic and histological structure. Squamous, glandular, transitional, and melanocytic components occur at this site, either alone, or in combination (Janicke DM. et al, 1996).

Most anal cancers in Europe and in the United States are SCC. These tumors come from the squamous cells that line the anal margin and most of the anal canal. SCC of the anal margin (perianal skin) are treated similarly to SCC of the skin elsewhere in the body (Martin FT. et al, 2009).

Tumors of the anal margin are usually well differentiated, in contrast to SCAC. Grading is subject to inter-observer variability, and considerable heterogeneity is seen in larger tumors. High-grade tumors have been thought to have a worse prognosis, but this has not been confirmed in multivariate analysis (Martin FT. et al, 2009).

SCAC can be either keratinized or non-keratinized depending on their location in relation to the dentate line. Importantly, both keratinizing and non-keratinizing tumors appear to have similar biology and prognosis (Martin FT. et al, 2009).

A small number of anal cancers are known as adenocarcinomas. These can develop in cells that line the upper part of the anus near the rectum, or in glands located under the anal mucosa that release their secretions into the anal canal. These anal adenocarcinomas behave quite differently from SCAC and are treated like rectal carcinomas (Tarazi R. et al, 1994).

### 1.3 CLINICAL PRESENTATION AND STAGING

Most patients with SCAC present with rectal bleeding. Diagnosis can be delayed because this bleeding is often ascribed to hemorrhoids. Other symptoms include rectal pain and/or mass sensation, occurring in approximately 30% of patients (Tanum G. et al, 1991). Twenty percent of patients have no symptoms at the time of diagnosis (Ryan DP. et al, 2000).

A tumor–node–metastasis (TNM) staging system for anal cancer has been developed by the American Joint Committee on Cancer and the International Union Against Cancer (Table 1). Because few tumors are surgically excised, the system is based on clinical factors with particular emphasis on tumor size, because this is known to be an important determinant of prognosis. Fifty

to sixty percent of patients present with T1–T2 lesions, for which the 5-year survival rate is 80–90%. A smaller proportion presents with T4 lesions, which have a 5-year survival rate of 50%. The incidence of nodal metastasis is approximately 10% at diagnosis but can increase to 60% for T4 lesions (Salmon RJ. et al, 1986).

**Table 1**

| | | | |
|---|---|---|---|
| T – Primary Tumour | | | |
| TX | Primary tumour cannot be assessed | | |
| T0 | No evidence of primary tumour | | |
| Tis | Carcinoma in situ | | |
| T1 | Tumour 2 cm or less in greatest dimension | | |
| T2 | Tumour more than 2 cm but not more than 5 cm in greatest dimension | | |
| T3 | Tumour more than 5 cm in greatest dimension | | |
| T4 | Tumour of any size invades adjacent organ(s), e.g., vagina, urethra, bladder (involvement of sphincter muscle(s) alone is not classified as T4) | | |
| N – Regional Lymph Nodes | | | |
| NX | Regional lymph nodes cannot be assessed | | |
| N0 | No regional lymph node metastasis | | |
| N1 | Metastasis in perirectal lymph node(s) | | |
| N2 | Metastasis in unilateral internal iliac and/or inguinal lymph node(s) | | |
| N3 | Metastasis in perirectal and inguinal lymph nodes and/or bilateral internal iliac and/or inguinal lymph nodes | | |
| M – Distant Metastasis | | | |
| MX | Distant metastasis cannot be assessed | | |
| M0 | No distant metastasis | | |
| M1 | Distant metastasis | | |
| **Stage Grouping** | | | |
| Stage 0 | Tis | N0 | M0 |
| Stage I | T1 | N0 | M0 |
| Stage II | T2 | N0 | M0 |
| | T3 | N0 | M0 |
| Stage IIIA | T1 | N1 | M0 |
| | T2 | N1 | M0 |
| | T3 | N1 | M0 |
| | T4 | N0 | M0 |
| Stage IIIB | T4 | N1 | M0 |
| | Any T | N2, N3 | M0 |
| Stage IV | Any T | Any N | M1 |

TNM staging system for anal cancer (sourcewww.iarc.fr/en/publications)

## 1.4 TREATMENT

### *1.4.1 Surgery*

Prior to the mid-1980s, the treatment of choice for anal cancer was APR, a procedure involving removal of the anus and rectum as well as their draining lymph nodes and resulting in a permanent colostomy. The 5-year overall survival (OS) rate after APR for anal carcinoma is in the range of 40–70%, with worse outcomes for those with larger tumors and nodal metastases (Martin FT. et al, 2009). APR is now reserved as salvage therapy for those individuals with persistent disease after combined chemoradiation.

### *1.4.2 Combined Chemoradiation*

Based on prior experience of fluoropyrimidines as radiosensitizers in different gastrointestinal malignancies, Nigro and colleagues in 1974 investigated the use of preoperative chemotherapy with 5-fluorouracil (5-FU) and mitomycin C (MMC) plus radiation therapy (RT) given in doses of 30 Gy in patients with SCAC (Nigro ND. et al, 1974). Interestingly, the first three patients treated achieved a complete pathologic response on evaluation of postsurgical specimens, and this resulted in the concept of anal sphincter preservation strategies. Subsequent patients series supported the use of chemoradiation protocols for anal sphincter preservation, reserving surgery for instances where residual disease persisted after combined modality chemoradiation therapy. These series showed 5-year OS of 70% and colostomy-free survival of 60% (Leichman L. et al, 1985; Doci R. et al, 1996).

These results were followed by several large randomized clinical trials, which confirmed the role of combined modality therapy and now form the basis of the current standard of care in management of SCAC.

Two European Phase III randomized clinical trials were conducted in late 1990s to evaluate the benefits of chemoradiation therapy versus RT alone. At the same time, two United States trials looked at the efficacy of different chemotherapeutic regimens in chemoradiation protocols in the treatment of SCAC (UKCCCR Anal Cancer Trial Working Party, 1996; Flam M. et al, 1996; Bartelink H. et al, 1997; Ajani JA. et al, 2008).

The United Kingdom Coordinating Committee on Cancer Research-based Anal Cancer (UKCCCR) Trial Working group compared RT alone versus RT, 5-FU, and MMC in a multicenter clinical trial in 1996 (UKCCCR Anal Cancer Trial Working Party, 1996). Five hundred eighty-five patients with stage T1 to T4 SCAC, with and without lymph node involvement, were randomized. Patients received either 45 Gy of RT in 20 or 25 fractions over 4 to 5 weeks (290 patients), or the same regimen of RT combined with 5-FU (1000 mg/m2 for 4 days or 750 mg/m2 for 5 days) by continuous intravenous (IV) infusion during the first and the final weeks of RT and MMC (12 mg/m2) on day 1 of the first course (295 patients). Clinical response was assessed at 6 weeks after initial treatment. Good responders received an additional boost of radiation and poor responders underwent salvage surgery. The main endpoint was local-failure rate (≥6 weeks after initial treatment); secondary endpoints were OS and cause-specific survival. Only 577 patients were eligible for assessment after randomization. Chemoradiation therapy resulted in less local failure rates (36% versus 59%, p≤0.0001) and decreased cancer-related risk of death (0.71, p=0.02). There was no significant

difference in OS between the two therapies at 36 months (65% versus 58%). Investigators concluded that chemoradiation therapy results in better survival and tumor control than RT alone (UKCCCR Anal Cancer Trial Working Party, 1996)

The European Organization for the Research and Treatment of Cancer (EORTC) randomized 110 patients to chemoradiation versus RT alone (Bartelink H. et al, 1997). They used 5-FU and MMC (750 mg/m2 and 15 mg, respectively, instead of 1000 mg/m2 and 12 mg in the UKCCCR Study). The addition of chemotherapy to RT resulted in a significant increase in the complete remission rate from 54% for RT alone versus 80% for combined modality chemoradiation therapy. Significant improvement in locoregional control and colostomy-free survival was noted ($p=0.02$ and $p=0.002$, respectively). The locoregional control rate improved up to 18% at 5 years, wheras the colostomy-free survival at that time increased to 32% with the addition of chemotherapy to RT. No significant difference in severe therapy related toxicity was noted, although anal ulcers were more frequently observed in the combined-treatment arm. The OS rate remained similar in both treatment arms. The results of this study supported the UKCCCR trial conclusions (Bartelink H. et al, 1997). While United Kingdom investigators were establishing the role of chemoradiation therapy in anal canal cancer, in North America investigators were concentrating on the evaluation of the need for MMC in combined modality therapy. As MMC is not a known radiosensitizer and is associated with higher renal, pulmonary, and bone marrow toxicities, U.S. investigators designed trials to compare chemoradiation therapy without MMC or replacing it with cisplatin (Flam M. et al, 1996).

The Radiation Therapy Oncology Group (RTOG) randomized 310 patients to 5-FU plus radiation or 5-FU and MMC plus radiation (Flam M. et al, 1996). 5-FU was infused at a rate of 1000 mg/m2 IV and MMC at 10 mg/m2 IV. Radiation doses ranged from 45 to 50.4 Gy. Post-treatment biopsies were positive in 15% of patients in the 5-FU arm versus 7.7% in the MMC arm (p=0.135). At 4 years, colostomy rates were lower (9% versus 22%; p=0.002), colostomy-free survival higher (71% versus 59%; p=0.014), and disease-free survival (DFS) higher (73% versus 51%; p=0.0003) in the MMC arm. A significant difference in OS was not observed at 4 years. Toxicity was found to be greater in the MMC arm (23% versus 7% grade 4 and 5 toxicity; p≤0.001). From these results, investigators reached the conclusion that despite more toxicity, MMC still plays an important role in combined modality therapy by reducing local recurrence and colostomy rates (Flam M. et al, 1996).

RTOG conducted a multicentre phase III trial comparing 5-FU plus MMC and RT versus treatment with 5-FU plus cisplatin and radiotherapy in 682 patients with SCAC (Ajani JA. et al, 2008). The MMC group received 5-FU (1000 mg/m2 on days 1 to 4 and 29 to 32) plus MMC (10 mg/m2 on days 1 and 29) and RT (45 to 59 Gy). The cisplatin group received fluorouracil (1000 mg/m2 on days 1 to 4, 29 to 32, 57 to 60, and 85 to 88) plus cisplatin (75 mg/m2 on days 1, 29, 57, and 85) and RT (45 to 59 Gy; start day=day 57). The median follow-up for all patients was 2.51 years. The 5-year disease-free and OS rate were not significantly different (p=0.17 and p=0.10). However, the 5-year locoregional recurrence, distant metastasis, and cumulative colostomy rates were significantly better for the MMC-based therapy group compared with the cisplatin-based treatment group (10% versus 19%; p=0.02). Severe

hematological toxicity was higher with MMC-based treatment ($p<0.001$) (Ajani JA. et al, 2008).

The two European trials have shown the superiority of chemoradiation over RT alone, whereas the two U.S. trials have endorsed the use of MMC in combined modality therapy, which significantly reduced local recurrence and colostomy rates, despite a higher toxicity rate. The authors concluded that MMC was an important part of combined chemoradiation for SCAC and this regimen has remained the standard of care.

### *1.4.3 Treatment Complications*

Chemoradiation therapy for SCAC can have both acute and chronic effects. Acute effects include diarrhea, mucositis, skin erythema and desquamation, and myelosuppression. Late complications, some of which necessitating surgery with or without colostomy, include anal ulcers, stenosis, fistulae, and necrosis. Reported late event rates following chemoradiation therapy for anal cancer are in the range of 3–16% (Clark MA. et al, 2006). The risk for these complications increases as a function of both total radiation dose and fraction size, with complications more frequent when fractions >2.5 Gy are used (Nigro ND. et al, 1983). As mentioned before, the UKCCCR trial failed to demonstrate a higher incidence of late effects with the use of combined chemoradiation compared with RT alone (UKCCCR Anal Cancer Trial Working Party, 1996). There is one study in the literature that specifically evaluates quality of life (QOL) after radiation alone or combined chemoradiation (Allal AS. et al, 1999). Allal and colleagues evaluated QOL in 41 patients (35 female and 6 male) who were alive at least 3 years after completing therapy for anal cancer. The study showed that patients treated

with RT with or without chemotherapy rated their QOL similar to that of the general population, with the exception of noting more frequent diarrhea. Interestingly, 50% of patients reported suboptimal anal function, whereas 71% reported that they were satisfied with their current function and only 7% would have considered APR as a potential alternative.

## 1.5 PERSISTENT OR RECURRENT DISEASE

Effects of chemoradiation on SCAC can be present after completion of treatment. Response is best assessed at least 6–8 weeks after completion. There is currently no consensus as to whether response should be assessed by physical examination alone or in combination with biopsy. It is also not clear whether biopsy should play a role in the management of those individuals with a CR.

There are few data available about predictors of local failure, but one retrospective study was identified (Renehan AG. et al, 2005). Renehan and colleagues evaluated outcomes of 254 patients with SCAC treated with either RT alone (n=127) or combined chemoradiation (n=127) between 1988 and 2000 at a hospital in the United Kingdom. Local failure occurred in 99 (39%) patients and the median time to failure was 20.4 months. Five-year local disease failure rates were significantly different between those patients receiving RT alone (52.5%) and those patients receiving combined chemoradiation (35.3%). For patients receiving RT alone, age, total radiation dose >50 Gy and higher T stage predicted local failure. Conversely, for patients receiving combined chemoradiation, no factor was predictive (Renehan AG. et al, 2005).

*1.5.1 Salvage APR*

The preferred treatment for persistent disease following combined modality therapy is APR. This surgery is radical and associated complications appear to be greater in patients undergoing the procedure after combined modality therapy (Clark MA. et al, 2004). Nilsson and colleagues retrospectively evaluated the outcomes of 35 Swedish patients (21 with persistent disease and 14 with recurrent disease) undergoing salvage APR following locoregional failure after combined modality therapy for SCAC (Nilsson PJ. et al, 2002). Thirteen patients developed perineal wound infection necessitating reoperation, and delayed wound healing (defined as healing time >3 months) occurred in 23 patients. Fifteen patients, 12 of whom underwent salvage APR for persistent disease, experienced secondary failure. The median survival duration after secondary failure was 19 (range, 1–78) months. In the UKCCCR trial, there were 29 patients who underwent salvage APR; 40% eventually relapsed (UKCCCRAnal Cancer Trial Working Party, 1996).

*1.5.2 Salvage Chemoradiation Therapy*

Salvage chemoradiation therapy for persistent disease has also been evaluated (Flam M. et al, 1996). In the Intergroup study evaluating the role of MMC, those patients with persistent disease received salvage 5-FU, cisplatin, and 9 Gy of external beam RT (EBRT). Of 29 patients treated in this manner, 10 continued to have persistent disease. Nine of these patients went on to salvage APR and six eventually recurred (Flam M. et al, 1996).

## 1.6 METASTATIC DISEASE

Metastatic disease develops in 10–17% of patients treated with chemoradiation therapy (UKCCCR Anal Cancer Trial Working Party, 1996; Bartelink H. et al, 1997). The most common site of distant metastasis is the liver. There are limited published data on the use of chemotherapy, particularly newer agents, to treat metastatic SCAC. Active agents include cisplatin plus 5-FU (Khater R. et al, 1986; Jaiyesimi IA. et al, 1993), carboplatin (Evans TR. et al, 1993), doxorubicin (Fisher WB. et al, 1978), and semustine (Zimm S. et al, 1981). Participation in a clinical trial should be discussed with all potentially eligible patients.

### *1.6.1 Targeted Therapy*

Because of the high cure rate of localized anal cancers from combined modality therapies, little is known for the treatment of patients who progress to have metastatic disease.

In searching for new therapeutic options for these forms of SCAC, and on the basis of activity demonstrated in other cancers with similar histology, targeted therapies with biological drugs have been recently considered.

Previously reports have supported the effective use of the EGFR-inhibitors cetuximab and panitumumab, two monoclonal antibodies (MoAbs) directed against the ligand binding domain of the epidermal growth factor receptor (EGFR), in colorectal (CRC) and non-small cell lung cancers (NSCLC) (Van den Eynde M. et al, 2011). In particular, for squamous cell variants, in 2006 cetuximab was approved by the Food and Drugs Administration (FDA) and by the European Medicine Agency (EMA) for the treatment of locally advanced head and neck squamous cell carcinoma (HNSCC) (in

combination with RT) and of recurrent/metastatic HNSCC (as single agent), after failure of platinum-based chemotherapy (Tejani MA. et al, 2010).

In addition, it has been shown that the inhibition of EGFR signaling is able to sensitize cells to radiation in HNSCC, supporting the use of cetuximab in combination with RT (Koukorakis G. et al, 2009; Dequanter D. et al, 2010).

Only few studies reported about the use of cetuximab in chemo-refractory patients with advanced SCAC (Phan LK. et al, 2007; Lukan N.et al, 2009; De Dosso S. et al, 2010; Saif M. et al, 2011). All these authors evidenced excellent response to cetuximab or panitumumab in a total of twelve patients with metastatic SCAC, after failure of cisplatin-based regimens.

The comprehension of biological mechanisms at the basis of cetuximab activity, especially concerning EGFR and the members of its downstream pathway, which are known to impair the efficacy of anti-EGFR therapies in metastatic CRC and in NSCLC, could help in a better identification of patients groups who can really benefit from this targeted therapy approach (Mao C. et al, 2009; Bardelli A. et al, 2010).

## 1.7 MOLECULAR BIOLOGY

SCAC shows a pattern of protein expression/gene deregulation similar to those observed in SCC arising in different organs (Holly EA. et al, 2001; Zhang J. et al, 2005). Only sporadic reports on the molecular biology of SCAC are currently available and most include fewer than 50 patients (Gervaz P. et al, 2006).

Current evidence suggests that HPV infection is necessary, but not sufficient, to promote progression of anogenital epithelium towards invasive cancer (Gervaz P. et al, 2006). Additional molecular changes include loss of

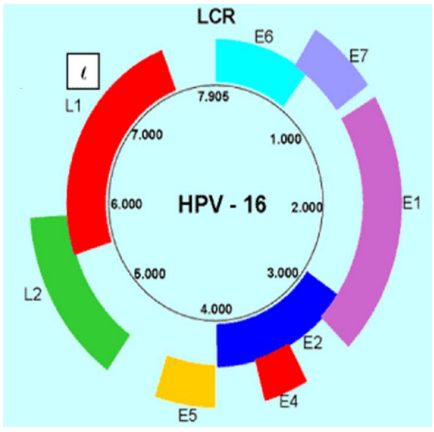
heterozygosity or epigenetic silencing through promoter hypermethylation of several tumor suppressor genes (Muleris M. et al, 1987; Heselmeyer K. et al, 1997; Gervaz P.et al, 2001; Gervaz P. et al, 2006).

Epidemiologic data have shown that human HIV infected patients are at highest risk of SCAC development (Gervaz P. et al, 2004).

### *1.7.1 HPV*

HPV genome is a 8 kb circular double-stranded DNA (Figure 2). It has two regulatory proteins (called early proteins) E1 and E2, three important oncogenes with growth-stimulating and transforming properties E5, E6 and E7, and two capsid proteins (called late proteins) L1 and L2. The oncogenes E6 and E7 are responsible for malignant transformation of infected cells and have been used by investigators to induce immortality in cell-lines and cell culture models. These two genes cause inactivation of two important tumor suppressor proteins: p53 and retinoblastoma (Dyson N. et al, 1989; Crook T. et al, 1991).

**Figure 2**




Schematic representation of HPV genome (source:http://www.colon.it/Condilomi_anali.html).

The L1 protein has been used to develop the HPV vaccine since it is the most conserved gene within the HPV genome.

HPV is a highly diverse group of viruses, ubiquitous in nature, with world-wide distribution. HPV has been detected from apparently normal skin and appears to have a highly variable latent period before detectable pathology (Antonsson A. et al, 2003). HPV is associated with lesions ranging from benign cutaneous warts, to recurrent laryngeal papillomatosis, to malignancies like cervical and anal cancers (Goon P. et al, 2008). Low- risk (LR) HPV types 6 and 11 are the most common HPV associated with genital warts and most cases of recurrent respiratory papillomatosis. High-risk (HR) HPV types 16 and 18 are most commonly associated with dysplasia.

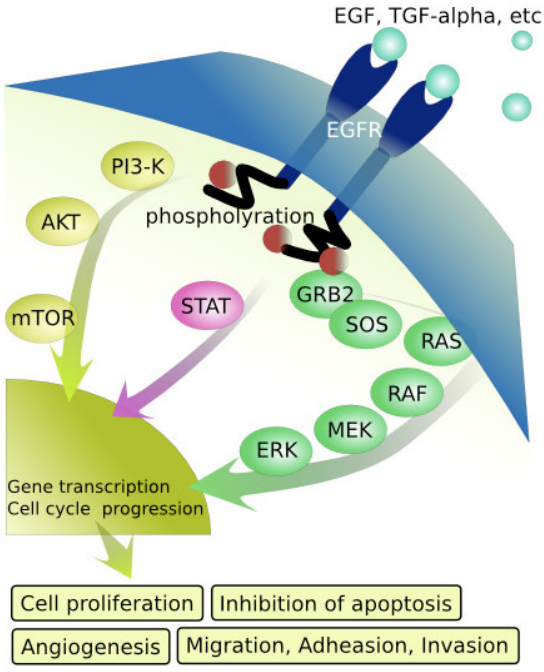
HPV infection has a high prevalence in sexually active population, yet most patients infected with HR HPV do not develop cancer. It has been reported that HR HPV is detected in 80-100% of anal cancer cases, and all various infected cases were shown to have integrated viral DNA (Holm R. et al, 1994; Gervaz P. et al, 2006). As HPV was detected in all phases of SCAC development (from anal intraepithelial neoplasia to invasive cancer), it has been suggested that HR oncogenic HPV subtypes represent the initiating event in anal epithelial transformation (Gervaz P. et al, 2006).

### *1.7.2 EGFR*

EGFR gene (also known as erbB1, HER1), located on the short arm of chromosome 7 (7p12), encodes for a glycoprotein of 170 kDa that consists of an extracellular receptor portion, a transmembrane region, and an intracellular domain with tyrosine kinase (TK) activity. EGFR binds several ligands, including EGF and transforming growth factor alpha (TGFα). After

ligand binding, EGFR can homodimerize or heterodimerize with other members of EGFR family (erbB2/HER2, erbB3/HER3, erbB4/HER4) triggering a cascade of events implicated in a wide range of cell functions, such as cell proliferation, migration, maturation and differentiation (Figure 3). EGFR-downstream signal transduction pathways are mainly represented by the RAS/RAF/MEK/ERK (MAP kinases) and the PI3K/PTEN/AKT pathways (Carpenter G. et al, 1990; Wells A., 1999).

**Figure 3**




EGFR activation and downstream signalling cascade (source: http://commons.wikimedia.org/wiki/File:EGFR_signaling_pathway.svg).

In cancer cells, EGFR receptor has been found to be either overexpressed on the membrane either mutated in the sequence coding for the TK domain (exon 18-21). These alterations result in a constitutive activation of EGFR, independent from the ligand binding, that causes the uncontrolled activation

of downstream signal transduction pathways, leading to tumor growth, metastasis, angiogenesis and inhibition of apoptosis (Baselga J., 2001).

EGFR is deregulated in a variety of solid malignant tumors (i.e. NSCLC, metastatic CRC, HNSCC), including SCAC, and is usually associated with disease progression and poor prognosis (Alvarez G. et al, 2006).

Given the myriad of downstream effects, its frequency of alteration and its correlation with prognosis, various approaches have been considered to inhibit EGFR, and, consequently, to inactivate its downstream pathways. These include gefitinib and erlotinib, two small molecules able to bypass the plasma-membrane and to bind to the TK mutated domain, and cetuximab and panitumumab, two MoAbs that bind to the extracellular domain. TK inhibitors (TKIs) are effective in patients with NSCLC and with locally advanced or metastatic pancreatic cancer, while MoAbs received FDA approval for the treatment of patients with locally advanced or metastatic HNSCC and CRC (Laskin JJ. et al, 2004; Baselga J. et al, 2005).

The deregulation of EGFR in SCAC has been investigated in very few works, with less than 50 investigated patients.

At protein level, EGFR overexpression has been detected by immunohistochemistry (IHC) in the great majority of cases. Authors used the same primary antibody (anti-EGFR mouse monoclonal IgG1 antibody) but different antigen-retrieval protocols, and observed EGFR positivity in 55% (21/33), 83% (36/43) and 100% (21/21) of SCAC patients, respectively (Le LH. et al, 2005; Alvarez G. et al, 2006; Van Damme N. et al, 2010).

At genetic level, EGFR was investigated by fluorescence in situ hybridization (FISH) in only two studies. Both reported an increase in EGFR gene copy number (copy number gain) due to polysomy of chromosome 7 in 34% (8/23)

and in 39% (7/18) of SCAC patients, respectively. Gene amplification has never been reported (Alvarez G. et al, 2006; Van Damme N. et al, 2010).

No correlation between EGFR protein overexpression as detected by IHC and gene copy number as detected by FISH has been revealed (Alvarez G. et al, 2006; Van Damme N. et al, 2010).

At sequence level, no mutation has been found in exon 18 to 21 of EGFR TK domain (in a cohort of 26 patients) (Van Damme N. et al, 2010).

The involvement of EGFR may have important therapeutic implications in the management of patients with SCAC, especially for those refractory to standard therapy. In fact, it has been recently reported that cetuximab in combination with irinotecan, is effective in tumor control in very few SCAC patients refractory to standard therapies, similarly to the results described in HNSCC (Phan LK. et al, 2007; Lukan N. et al; 2009, De Dosso S. et al, 2010; Saif MW. et al, 2011). In contrast, the use of TKI in SCAC has never been reported.

### *1.7.3 Members of EGFR downstream pathways*

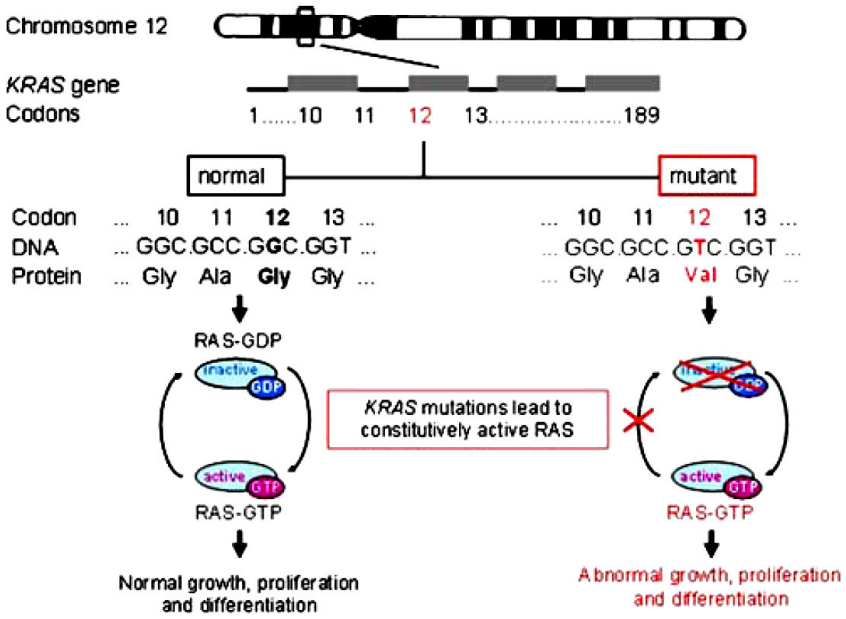
A rapidly growing body of knowledge has indicated that proliferation in many tumors is driven by constitutive activation of signaling pathways downstream to EGFR, as discussed before. Such close interactions between these pathways may provide escape mechanisms that allow tumors to circumvent a pathway that has been pharmacologically blocked. The interlinked RAS–MAPK and PI3K signaling pathways play an important role in tumorigenesis via phosphorylation of various proteins and transcription factors that directly control cell growth, differentiation, and apoptosis.

It has been recently demonstrated that mutation in KRAS, BRAF, or PIK3CA oncogenes are important predictive markers of resistance to cetuximab or panitumumab treatment in mCRC. Rare studies investigated these markers in SCAC (Patel H. et al, 2007; Lukan N. et al, 2009; Zampino MG. et al 2009; van Damme N. et al, 2010).

*KRAS*. KRAS is a member of the rat sarcoma virus (ras) gene family of oncogenes (including HRAS, and NRAS), located on chromosome 12, encoding for the guanosine bis/tris phosphate (GDP/GTP)–binding protein RAS, that acts as a self-inactivating intracellular signal transducer (Raaijmakers JH. et al, 2009). RAS proteins normally cycle between active GTP-bound (RAS-GTP) and inactive GDP-bound (RAS-GDP) conformations (Figure 4). RAS proteins are activated by guanine nucleotide exchange factors (GEFs) which are recruited to protein complexes at the intracellular domain of activated receptors. Signaling is terminated when RAS-GTP is hydrolyzed to the RAS-GDP inactive complex by GTPase-activating proteins (GAPs) (Van Krieken H. et al, 2008; Raaijmakers JH. et al, 2009). After binding and activation by GTP, RAS recruits the protein encoded by RAF oncogene, which phosphorylates Mitogen-Activated Protein Kinase Kinase-1 (MAP2K-1) and MAP2K-2, thus initiating the MAPK signaling that ultimately leads to the expression of proteins playing important roles in cell growth, differentiation, and survival. Under physiological conditions, RAS-GTP levels are tightly controlled by the counterbalancing activities of GEFs and GAPs.
KRAS mutations are one of the most common gene alterations in human cancer. KRAS mutations result in RAS proteins that are permanently in the

active GTP-bound form due to defective intrinsic GTPase activity and resistance to GAPs (Figure 4).

**Figure 4**




Ras activation. Normal condition on the left. Constitutive activation caused by oncogenic point mutations on the right (Van Krieken H. et al, 2008).

Unlike wild-type RAS proteins which are inactivated after a short time, the aberrant proteins are able to continuously activate signaling pathways in the absence of any upstream stimulation of EGFR/HER receptors. There are a limited number of mutations in the KRAS gene, and altogether more than 90% involve two codons (12 and 13). Of these, the most frequent alterations are detected in codon 12 (about 80% of all reported KRAS mutations). Codons 12 and 13 somatic missense mutations lead to single amino acid substitutions and are generally independent from EGFR mutations (Kosaka T. et al, 2004). Mutations in other positions, such as codons 61 and 146,

have also been reported, but these make up less than 10% of mutations (Edkins S. et al, 2006).

In SCAC, KRAS mutations have been described only in 2 out of 7 patients with metastatic disease treated with cetuximab (Lukan N. et al, 2009). In other two cohorts of unselected SCAC, KRAS was always wild type (i.e. 0/26 and 0/30 investigated cases, respectively) (Zampino MG. et al 2009; van Damme N. et al, 2010).

*BRAF*. BRAF gene, located on chromosome 7, encodes for a RAS effector belonging to the RAF family of Ser-Thr kinase proteins. BRAF gene product is recruited to the plasma membrane upon binding to RAS-GTP, and represents a key point in the signal transduction through the MAP kinase pathway.

BRAF sequence contains three conserved regions: CR1, that encodes for the putative zinc finger domain, CR2, where several Ser-Thr-rich regions are located, and CR3, which corresponds to the kinase domain. The two major regulatory sites are Thr599 and Ser602, phosphorylated by RAS.

BRAF is the only RAF protein found to be frequently mutated in cancer. Mutations were identified in approximately 50% of melanoma, and in a smaller percentage of other tumors, including thyroid, colonic and ovarian carcinomas, and some sarcomas (Michaloglou C. et al, 2008). All mutations are represented by activating missense point mutations clustered in exons 11 and 15. In particular, the most common oncogenic BRAF mutation, occurring in more than 90% of cases, corresponds to a T>A transversion at position 1799 of BRAF sequence, resulting in the Valine to Glutamate substitution at position 600 of the protein (V600E) within the kinase domain, thus mimicking

the phosphorylation of Thr599 and Ser602. This change leads therefore to a mutated BRAF protein with elevated kinase activity, able to constitutively activate MAPK pathway (Davies H. et al, 2002).

At the moment, BRAF gene mutations have never been investigated in SCAC.

*PI3K.* Phosphatidylinositol 3-kinases (PI3Ks) belong to the lipid kinases family that regulates the signal transduction (Vivanco I. et al, 2002). PI3K proteins are constituted by catalytic and adaptor/regulatory subunits variants encoded by separate genes, are originated by alternative splicing, and are activated downstream of several TK receptors like EGFR, HER2, IGF1R, cKIT, PDGFR and MET, that, directly or through adaptor proteins, bind to and activate PI3Ks. Activation of PI3Ks results in the production of the second messenger phosphatidylinositol (PI) 3,4,5 trisphosphate (PIP3) from PI 4,5 bisphosphate (PIP2). PIP3, through AKT activation, drives various downstream pathways involved in the regulation of several functions including cellular growth, transformation, adhesion, apoptosis, survival and motility (Yuan TL. et al, 2008).

PI3Ks are antagonized by the phosphatase PTEN (Phosphatase and TENsin homolog deleted on chromosome 10) that catalyzes the opposite reaction. Constitutive activation and overexpression of PI3Ks (and inactivation of PTEN) results in enhanced PI3K signalling leading to oncogenic cellular transformation and cancer. Only PI3K proteins that contain the catalytic subunit p110α, and its associated regulatory subunit p85, are involved in tumorigenesis (Samuels Y. et al, 2010). The p110α subunit is encoded by PIK3CA, a 34 kb gene located on chromosome 3 (3q26.3) consisting of 20

exons and coding for a 124 kDa size protein. Hyperactivating PIK3CA mutations have been identified in several tumors, including breast, endometrial, urinary tract, ovarian, brain and gastric cancers (Yuan TL. et al, 2008; Samuels Y. et al, 2010).

The large majority of PIK3CA mutations cluster in two conserved regions, the helical domain, encoded by exon 9, and the kinase domain, encoded by exon 20. The hotspot mutations E542K and E545K (in exon 9), and H1047R (in exon 20), are non-synonymous missense mutations that confer a constitutive kinase activity to the protein. Rare mutations may also occur in exons 6 and 7 (Karakas B. et al, 2006).

In SCAC, PIK3CA was investigated in only one study. Five coding sequence mutations were found out of 127 patients (4%). All were aminoacid substitutions: one in exon 9 (E545K) and 4 in exon 20 (3 H1047R and 1 H1047L) (Patel H. et al, 2007).

# 2 AIM OF THE STUDY

Aim of the present study was to characterize the pathway of EGFR, by analyzing EGFR gene status by FISH and KRAS, BRAF and PIK3CA mutations by sequencing in a large cohort of SCAC patients, for a possible introduction of anti-EGFR MoAb treatments, able to overcome the severe effects of ablative surgery in persistent or recurrent disease.

## 2.1 RATIONALE

In the era of targeted therapies, novel anti-EGFR biological drugs have entered into clinical practice. Anti-EGFR MoAbs, such as cetuximab and panitumumab, have been FDA and EMA approved in the treatment of mCRC and HNSCC showing efficacy in around 10-30% of patients (Tejani MA. et al, 2010; Van den Eynde M. et al, 2011).

It has been recently demonstrated that a proficient molecular profile is fundamental in predicting response to anti-EGFR agents. In particular, experience in mCRC revealed that EGFR deregulation, both at protein and genetic level, is a promising approach to predict response. But, since at the moment IHC is not considered suitable for the investigation of EGFR protein expression, because many pre-analytical and analytical factors could bias the results, and therefore patients selection, FISH seems to be a reliable tool for the determination of EGFR gene copy number and the prediction of efficacy to anti-EGFR drugs (Atkins D, et al, 2004; Langner C, et al, 2004; Moroni M. et al, 2005; Kersting C, et al, 2006; Frattini M. et al, 2007). However,

mutations occurring in EGFR-downstream members, such as KRAS, BRAF and PIK3CA genes, are able to impair response to anti-EGFR therapy and their role in conferring resistance has appeared, at least in mCRC (Moroni M. et al, 2005; Lievre A. et al, 2006; Benvenuti S. et al, 2007; Frattini M. et al, 2007).

Anecdotic studies investigating the use of the MoAb cetuximab in SCAC showed efficacy and disease control in very few patients with advanced and refractory forms (Phan LK. et al 2007; Lukan N.et al, 2009; De Dosso S. et al, 2010; Saifi M. et al, 2011). Unfortunately, deregulation of EGFR and of members of its downstream pathways have been poorly characterized in SCAC.

# 3 PATIENTS AND METHODS

## 3.1 PATIENTS

We retrospectively analyzed a cohort of 93 patients with histologically confirmed SCAC. The study was approved by the Institutional Ethical Committee of the Institute of Pathology, Locarno, Switzerland.

Forty-four patients were collected at the Institute of Pathology, Locarno, Switzerland, 27 at the University School of Medicine, Novara, Italy, 12 at the Civil Hospital, Legnano, Milan, Italy, and 10 at the Medicine Department, Modena Hospital, Modena, Italy.

All histological sections (from biopsies and excisions) were centrally reviewed by two expert pathologists who confirmed the diagnoses of SCAC.

Patients were operated between 1997 and 2010. Sixty-eight (73%) patients were female and twenty-five (27%) were male. Age at diagnosis ranged from 24 to 95 years (median age: 64), with 16 patients (17%) younger than 50 years, and 6 patients (6%) younger than 40 years. Sample size ranged from 0.2 cm to 5 cm in diameter (median: 0.7 cm). Considering patients for which data were available, 35/82 (43%) were classified as grade (G) 3, 37/82 (45%) as G2 and 10/82 (12%) as G1. The majority of tumors (60%) were keratinized.

The clinical-pathological features of SCAC patients are detailed in Table 2.

**Table 2**

| Characteristics | No | % |
|---|---|---|
| Sex | | |
| Male | 25 | 27 |
| Female | 68 | 73 |
| Age (years) | | |
| >50 | 77 | 83 |
| <50 | 16 | 17 |
| Tumor Grade | | |
| G1 | 10 | 12 |
| G2 | 37 | 45 |
| G3 | 35 | 43 |
| *missing* | *11* | - |
| Keratinization | | |
| yes | 30 | 60 |
| no | 20 | 40 |
| *missing* | *43* | - |

Clinical and histopathological data of investigate SCAC patients.

### 3.2 MOLECULAR ANALYSES

All the analyses were performed on formalin-fixed (10% buffered) paraffin-embedded (FFPE) tumor specimens.

FFPE tumor blocks were reviewed for quality and tumor content by analyzing detailed morphology of haematoxylin and eosin stained tissue sections of each blocks.

A single representative block from each case, containing at least 70% of neoplastic cells, was selected for FISH, HPV, and sequencing analyses.

To minimize cross contamination, the microtome blade was changed and the microtome surface was cleaned after each sample was sectioned.

For HPV and sequencing analyses, tumor macrodissection was performed when necessary to minimize the presence of non-neoplastic tissues.

*3.2.1 HPV analysis*

After deparaffinization, DNA was extracted using the QIAamp Mini kit (Qiagen) according to manufacturer's instructions.

HPV detection was performed using the INNO-LiPA HPV genotyping extra amp kit (Innogenetics). This test is a polymerase chain reaction (PCR)-based line hybridization assay that utilizes a cocktail of biotinylated consensus primers (SPF10) to amplify a portion of the L1 ORF of 15 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82), 7 LR HPV types (6, 11, 40, 43, 44, 54, 70) and 5 probable HR types (pHR, 26, 53, 66, 69/71, 74).

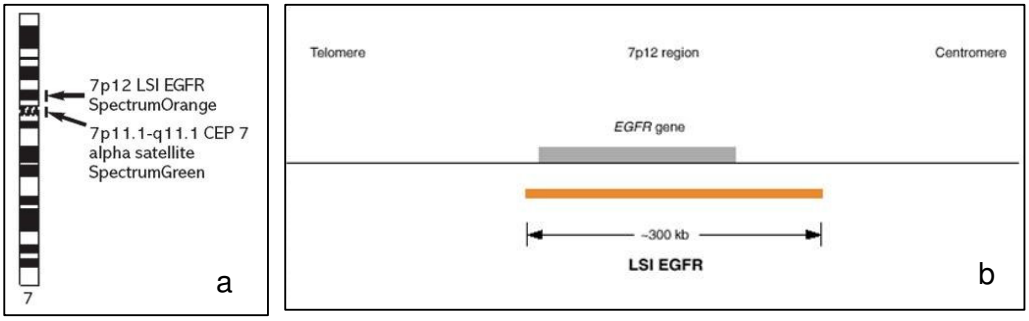
Four µl of DNA solution (25 ng/µl) was used for the PCR assay in a final volume of 25 µl using AmpliTaq Gold. PCR reaction consisted of 40 cycles (30 sec of denaturation at 94°C followed by 45 sec of annealing at 52°C and 45 sec of extension at 72°C). The PCR product was then denatured, and a 10 µl aliquot was hybridized on nitrocellulose strips onto which HPV type-specific oligonucleotides were already bound. After 60 min at 49°C, the PCR product bound to a specific probe was detected by an alkaline phosphatase-streptavidin conjugate and colorimetric detection. The reading of the hybridized strips was performed by comparison with standard control strips, following standardized criteria (Safaeian M. et al, 2007).

*3.2.2 FISH*

EGFR gene status evaluation was realized on 3 µm thick tissue sections that were treated using Paraffin Pretreatment kit II (Abbott Molecular) according to manufacturer's instructions. Dual-color FISH assay was performed using LSI EGFR/CEP7 probes (Abbott Molecular). The LSI EGFR probe is labeled in SpectrumOrange and covers an approximately 300 kb region that contains

the entire EGFR gene at 7p12. The CEP7 probe, labeled in SpectrumGreen, hybridizes to the alpha satellite DNA located at the centromere of chromosome 7 (7p11.1– q11.1) (Figure 5).

**Figure 5**




Schematic representation of LSI EGFR/CEP7 probe set (Abbott Molecular). A: probes map on chromosome 7, B: EGFR probe construction.

Target sections and probes were co-denatured at 75°C for 5 min and allowed to hybridize overnight at 37°C. A post-hybridization stringency wash was carried out in a water bath at 72°C for 5 min. After washing twice and drying at room temperature for 10 min, slides were mounted with 406-diamidino-2-phenylindole (DAPI II; Abbott Molecular). FISH signals were evaluated with a fluorescent automated microscope (Zeiss Axioplan 2 Imaging, Zeiss) equipped with single and triple band pass filters. Image for documentation were captured using an AxioCam camera (Zeiss Axiocam MRm) and processed using the AxioVision system (Zeiss). A minimum of 100 morphology-clear, non-overlapping nuclei from at least 8-10 different areas were scored for each patient.

For cases in which only a biopsy was available, all the analyzable nuclei were evaluated.

Patients were classified according to two different approaches, using both descriptive criteria (developed on the basis of cytogenetic classification) and a scoring system based on gene copy number gain (adopted for EGFR FISH interpretation in NSCLC, namely Colorado Scoring System) (Martin V. et al, 2009; Varella Garcia M. et al, 2009; Martin V. et al, 2012).

In details, the descriptive criteria were based on the highlighted abnormalities and the percentage of cells involved: patients exhibiting one balanced copy of EGFR gene and chromosome 7 centromere in >50% of tumor cells were classified as loss (loss); 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 ≥40% of cells were classified as low polysomic (LP) or high polysomic (HP), respectively; patients with a ratio (R) EGFR gene/chromosome 7 centromere >2 in ≥10% of cells were classified as EGFR amplified (A). In parallel, patients carrying ≥4 copies of EGFR in ≥40% of cells or gene amplification were considered as FISH positive (FISH+), while those with ≥4 copies in <40% of cells were classified as FISH negative (FISH-) (Varella Garcia M., 2006; Martin V. et al, 2009; Varella Garcia M. et al, 2009; Martin V. et al, 2012).

*3.2.3 Mutational analysis*

After deparaffinization, genomic DNA was extracted using the QIAamp Mini kit (Qiagen) according to manufacturer's instructions.

KRAS (exon 2), BRAF (exon 15) and PIK3CA (exons 9 and 20) mutations were detected by direct sequencing on genomic DNA as already reported (Frattini M. et al, 2004 (a); Frattini M. et al, 2007; Di Nicolantonio F. et al, 2008; Sartore-Bianchi A. et al, 2009; Martin V. et al, 2012). KRAS exon 2

includes codons 12 and 13, BRAF exon 15 includes codon 600, PIK3CA exon 9 includes codons 542 and 545 and PIK3CA exon 20 includes codon 1047. All these codons represent sites where the large majority of oncogenic mutations occur (Davies H. et al, 2002; Samuels Y. et al, 2004; Frattini M. et al, 2004 (b)). The nucleotide sequence corresponding to every exon was amplified from tumor-extracted genomic DNA by PCR, purified (Microcon YM-50, Millipore) and directly sequenced. Times, temperatures and cycles of PCR reactions for each gene are detailed in Table 3. The list of primers used for mutational analyses is reported in Table 4. All samples were subjected to automated sequencing by ABI PRISM 3130 (Applied Biosystems). All mutated cases were confirmed at least twice starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands. Direct sequencing has a sensitivity of about 20%.

**Table 3**

| | KRAS | | | BRAF | | | PIK3CA exon 9 | | | PIK3CA exon 20 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| step | T (°C) | time | cycles | T (°C) | time | cycles | T (°C) | time | cycles | T (°C) | time | cycles |
| STABILIZATION | 50 | 2' | - | 50 | 2' | - | 50 | 2' | - | 50 | 2' | - |
| INITIAL DENATURATION | 95 | 10' | - | 95 | 10' | - | 95 | 10' | - | 95 | 10' | - |
| DENATURATION | 95 | 15" | | 95 | 15" | | 95 | 30" | | 95 | 30" | |
| HYBRIDIZATION | 55 | 30" | 40 | 52 | 30" | 45 | 56 | 30" | 40 | 55 | 30" | 40 |
| EXTENSION | 72 | 30" | | 72 | 30" | | 72 | 30" | | 72 | 30" | |
| FINAL EXTENSION | 72 | 3' | - | 72 | 3' | - | 72 | 10' | - | 72 | 10' | - |

List of PCR conditions. T: temperature.

**Table 4**

| gene | exon | forward | reverse |
|---|---|---|---|
| KRAS | 2 | TGGTGGAGTATTTGATAGTGTA | CATGAAAATGGTCAGAGAA |
| BRAF | 15 | TCATAATGCTTGCTCTGATAGGA | GGCCAAAAATTTAATCAGTGGA |
| PIK3CA | 9 | GGGAAAAATATGACAAAGAAAGC | CTGAGATCAGCCAAATTCAGTT |
| PIK3CA | 20 | CTCAATGATGCTTGGCTCTG | TGGAATCCAGAGTGAGCTTTC |

List of primers used for PCR reactions.

### *3.2.4 Statistical analysis*

The two-tailed Fisher's exact test was used to calculate p values for the association between clinical-pathological and molecular data. The level of significance was set at p=0.05.

# 4 RESULTS

## 4.1 HPV RESULTS

HPV test was performed on 91 samples. In two cases material was not sufficient for the analysis. Almost all patients (87/91, 96%) were positive for HPV infection (HPV+), only 4 (4%) patients were negative (HPV-).

The great majority of cases (79/87, 91%) were HR type, 2 (2%) cases were probable HR type, 5 (6%) cases showed a mixed HR and LR type and 1 (1%) case was LR. Among HR type, type 16 was predominant (70/81, 86%). Remaining patients showed types 18, 31, 33, 35, 45, 58, or a combination of different types (Table 5).

**Table 5**

| HPV | No | % | No |
|---|---|---|---|
| negative | 4 | 4 | |
| positive | 87 | 96 | |
| HPV 6 LR | | | 1 |
| HPV 16 HR | | | 70 |
| HPV 18 HR | | | 2 |
| HPV 26 pHR | | | 2 |
| HPV 31 HR | | | 1 |
| HPV 33 HR | | | 2 |
| HPV 35 HR | | | 1 |
| HPV 45 HR | | | 1 |
| HPV 58 HR | | | 1 |
| HPV 18 HR , 58 HR | | | 1 |
| HPV 11 LR, 16 HR | | | 2 |
| HPV 6 LR, 16 HR, 45 HR | | | 2 |
| HPV 6 LR, 11 LR, 16 HR | | | 1 |
| *missing* | *2* | - | |

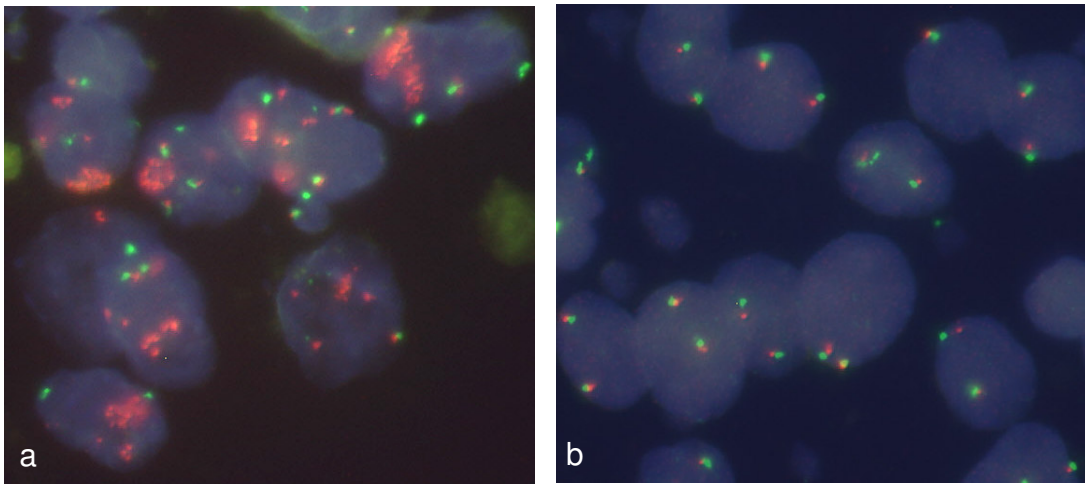
HPV types distribution in investigated SCAC patients. LR: low risk HPV, HR: high risk HPV, pHR: probable HR HPV.

## 4.2 EGFR FISH RESULTS

FISH was successful in 90 cases; 3 samples were not suitable for FISH analysis due to tissue fixation. Considering the classical cytogenetic classification, 4 cases (4%) were considered as A. Of these, three patients presented high level of EGFR gene amplification (R>10) with large clusters of signals in all cells (Figure 6a), whereas one patient showed a low level of amplification (2<R<3) in different cellular foci, corresponding to 50% of cells of the entire section. Fifteen cases (17%) were classified as HP, 32 cases (36%) as LP, 37 cases (41%) as D (Figure 6b) and 2 cases (2%) as loss (Table 6).

On the basis of gene copy number gain, 33 patients (37%) were grouped as FISH+ and 57 (63%) as FISH- (Table 6).

**Figure 6**




EGFR FISH assay on SCAC patients. A: EGFR gene amplification (R>2 between red/EGFR gene and green/chromosome 7 centromere signals). B: EGFR disomic pattern.

**Table 6**

| EGFR FISH | No | % |
|---|---|---|
| class | | |
| loss | 2 | 2 |
| D | 37 | 41 |
| LP | 32 | 36 |
| HP | 15 | 17 |
| A | 4 | 4 |
| *missing* | *3* | - |
| group | | |
| FISH- | 57 | 63 |
| FISH+ | 33 | 37 |
| *missing* | *3* | - |

EGFR FISH results by class (conventional cytogenetical classification) and by group (on the basis of gene copy number gain). A: amplification, D: disomy, FISH+: patients carrying ≥4 copies of EGFR in ≥40% of cells or gene amplification, FISH-: patients with ≥4 copies in <40% of cells, loss: loss of chromosome 7, HP: high polysomy, LP: low polysomy.

## 4.3 MUTATIONAL ANALYSIS RESULTS

### *4.3.1 KRAS SEQUENCING*

KRAS analysis was successful in 91 patients; sequences of two patients were not evaluable due to poor quality of DNA.

Mutations in KRAS gene were found in 4 patients (4%) (Table 7). All mutations were represented by classical high frequency alterations, the G12D change (GGT→GaT, Gly→Asp) in 3 cases (Figure 7a) and the G12V mutation (GGT→GtT, Gly→Val) in the remaining one.

### *4.3.2 BRAF SEQUENCING*

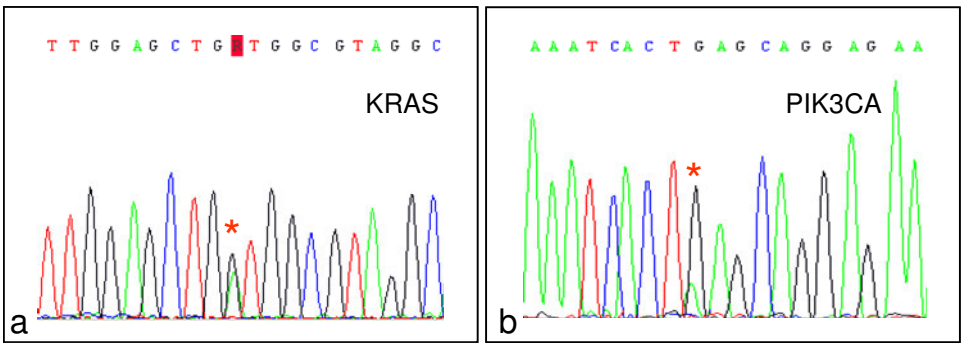
BRAF analysis gave analysable results in 90 patients; three patients were not evaluable. No mutations were found in BRAF gene (Table 7).

### *4.3.3 PIK3CA SEQUENCING*

PIK3CA sequencing was successful in 89 patients; four patients were not evaluable. Mutations in PIK3CA were found in 13 cases (15%) (Table 7).

Ten mutations occurred in exon 9 and 3 in exon 20. Mutations in exon 9 involved codon 545 in 7 cases, codon 546 in 2 cases and codon 542 in one case. At codon 545 all mutations corresponded to the transition G→A in the first base of the codon (GAG→aAG, Glu→Lys, E545K) (Figure 7b). Mutations in codon 546 involved the first base with the substitution C→A in one case (CAG→aAG, Gln→Lys, Q546K) and C→G in another one (CAG→gAG, Gln→Glu, Q546E); at codon 542 the mutation was present in the first base (GAA→aAA Glu→Lys, E542K). In exon 20, two mutations occurred at the classical codon 1047 (CAT→CgT, His→Arg, H1047R) and the other one at codon 1048 (CAT→tAT, His→Tyr, H1048Y).

**Figure 7**




Sequences profiles in SCAC patients.

A: presence of mutation in KRAS gene: asterisk indicates the G12D change (GGT→GaT, Gly→Asp).

B: presence of mutation in PIK3CA exon 9: asterisk indicates the E545K change (GAG→aAG, Glu→Lys, E545K).

**Table 7**

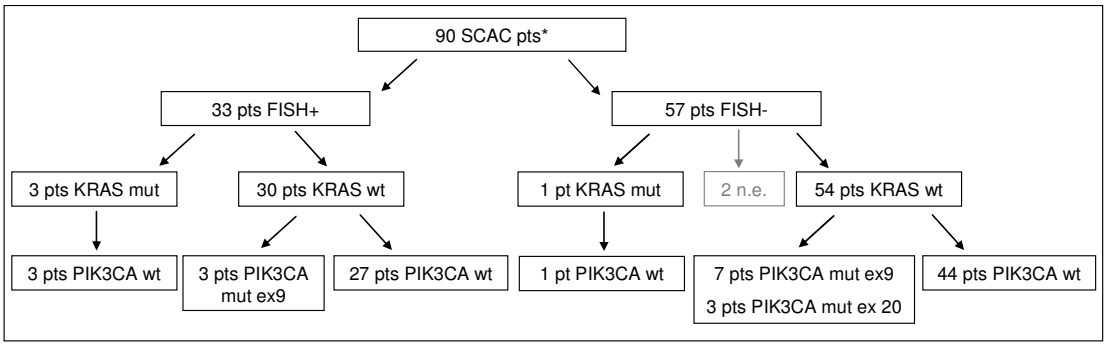
| mutational analysis | No | % |
|---|---|---|
| KRAS | | |
| wt | 87 | 96 |
| mut | 4 | 4 |
| *missing* | *2* | - |
| BRAF | | |
| wt | 90 | 100 |
| mut | 0 | 0 |
| *missing* | *3* | - |
| PIK3CA | | |
| wt | 76 | 85 |
| mut | 13 | 15 |
| *missing* | *4* | - |

Sequencing results for KRAS, BRAF and PIK3CA genes. Mut: mutation, wt: wild-type.

## 4.4 EGFR AND DOWNSTREAM MEMBERS RELATIONSHIP

Overall, EGFR-downstream members were altered in 17 patients (18%). No patients exhibited concomitant mutations in KRAS and PIK3CA genes (Figure 8).

**Figure 8**




Algorithm of comprehensive cytogenetical and molecular results.
Ex: exon, mut: mutation, n.e.: KRAS not evaluable, pts: patients, wt: wild-type, *: SCAC patients with successful FISH.

Among the 33 FISH+ patients, 6 (18%) patients showed a mutation in KRAS or PIK3CA concomitant to EGFR gene copy number gain (Table 8).

In details, three patients had a FISH+ profile and KRAS mutation (cases #29, #37,#57), and three patients had a FISH+ profile and PIK3CA mutation in exon 9 (cases #10, #15, #82).

**Table 8**

| # | FISH EGFR | | KRAS | BRAF | PIK3CA EX 9 | PIK3CA EX 20 |
|---|---|---|---|---|---|---|
| | class | status | | | | |
| 1 | D | FISH- | WT | WT | E545K | WT |
| 2 | D | FISH- | WT | WT | WT | WT |
| 3 | LP | FISH+ | WT | WT | WT | WT |
| 4 | D | FISH- | WT | WT | Q546K | WT |
| 5 | n.e. | n.e. | WT | n.e. | WT | WT |
| 6 | LP | FISH- | WT | WT | WT | WT |
| 7 | LP | FISH- | WT | WT | WT | WT |
| 8 | D | FISH- | WT | WT | WT | H1048Y |
| 9 | LP | FISH+ | WT | WT | WT | WT |
| 10 | LP | FISH+ | WT | WT | E545K | WT |
| 11 | LP | FISH- | WT | n.e. | WT | WT |
| 12 | loss | FISH- | WT | WT | WT | WT |
| 13 | LP | FISH- | WT | WT | WT | WT |
| 14 | LP | FISH- | WT | WT | Q546E | WT |
| 15 | HP | FISH+ | WT | WT | E545K | WT |
| 16 | D | FISH- | WT | WT | WT | WT |
| 17 | D | FISH- | WT | WT | WT | WT |
| 18 | D | FISH- | WT | WT | WT | WT |
| 19 | D | FISH- | WT | WT | WT | H1047R |
| 20 | D | FISH- | WT | WT | WT | WT |
| 21 | LP | FISH- | WT | WT | WT | WT |
| 22 | A | FISH+ | WT | WT | WT | WT |
| 23 | D | FISH- | WT | WT | WT | WT |
| 24 | LP | FISH+ | WT | WT | WT | WT |
| 25 | D | FISH- | WT | WT | WT | WT |
| 26 | HP | FISH+ | WT | WT | WT | WT |
| 27 | D | FISH- | WT | WT | WT | WT |
| 28 | D | FISH- | WT | WT | E545K | WT |
| 29 | HP | FISH+ | G12D | WT | WT | WT |
| 30 | LP | FISH+ | WT | WT | WT | WT |
| 31 | HP | FISH+ | WT | WT | WT | WT |
| 32 | LP | FISH- | WT | WT | WT | WT |
| 33 | D | FISH- | WT | WT | WT | WT |
| 34 | D | FISH- | WT | WT | WT | WT |
| 35 | D | FISH- | WT | WT | WT | WT |
| 36 | D | FISH- | WT | WT | WT | WT |
| 37 | HP | FISH+ | G12V | WT | WT | WT |
| 38 | HP | FISH+ | WT | WT | WT | WT |
| 39 | LP | FISH- | n.e. | n.e. | n.e. | n.e. |
| 40 | D | FISH- | WT | WT | WT | WT |

Molecular profile of SCAC patients. A: amplification, D: disomy, FISH+: ≥4 copies of EGFR in ≥40% of cells or amplification, FISH-: ≥4 copies in <40% of cells, loss: loss of chromosome 7, HP: high polysomy, LP: low polysomy, n.e.: not evaluable, wt: wild-type.

**Table 8** ***(continuation)***

| # | FISH EGFR | | KRAS | BRAF | PIK3CA EX 9 | PIK3CA EX 20 |
|---|---|---|---|---|---|---|
| | class | status | | | | |
| 41 | D | FISH- | WT | WT | WT | WT |
| 42 | LP | FISH- | WT | WT | WT | WT |
| 43 | HP | FISH+ | WT | WT | WT | WT |
| 44 | LP | FISH+ | WT | WT | WT | WT |
| 45 | D | FISH- | WT | WT | WT | WT |
| 46 | LP | FISH+ | WT | WT | WT | WT |
| 47 | LP | FISH+ | WT | WT | WT | WT |
| 48 | D | FISH- | WT | WT | WT | WT |
| 49 | loss | FISH- | WT | WT | WT | WT |
| 50 | HP | FISH+ | WT | WT | WT | WT |
| 51 | HP | FISH+ | WT | WT | WT | WT |
| 52 | HP | FISH+ | WT | WT | WT | WT |
| 53 | LP | FISH- | WT | WT | WT | WT |
| 54 | A | FISH+ | WT | WT | WT | WT |
| 55 | D | FISH- | WT | WT | E545K | WT |
| 56 | D | FISH- | WT | WT | WT | WT |
| 57 | LP | FISH+ | G12D | WT | n.e. | n.e. |
| 58 | LP | FISH- | WT | WT | n.e. | n.e. |
| 59 | D | FISH- | n.e. | WT | n.e. | n.e. |
| 60 | D | FISH- | WT | WT | WT | WT |
| 61 | LP | FISH- | WT | WT | WT | WT |
| 62 | A | FISH+ | WT | WT | WT | WT |
| 63 | D | FISH- | WT | WT | WT | WT |
| 64 | D | FISH- | WT | WT | WT | WT |
| 65 | LP | FISH- | WT | WT | WT | H1047R |
| 66 | LP | FISH+ | WT | WT | WT | WT |
| 67 | D | FISH- | WT | WT | E545K | WT |
| 68 | LP | FISH- | WT | WT | WT | WT |
| 69 | HP | FISH+ | WT | WT | WT | WT |
| 70 | D | FISH- | G12D | WT | WT | WT |
| 71 | D | FISH- | WT | WT | WT | WT |
| 72 | D | FISH- | WT | WT | WT | WT |
| 73 | D | FISH- | WT | WT | WT | WT |
| 74 | LP | FISH- | WT | WT | E542K | WT |
| 75 | D | FISH- | WT | WT | WT | WT |
| 76 | LP | FISH- | WT | WT | WT | WT |
| 77 | D | FISH- | WT | WT | WT | WT |
| 78 | LP | FISH+ | WT | WT | WT | WT |
| 79 | D | FISH- | WT | WT | WT | WT |
| 80 | D | FISH- | WT | WT | WT | WT |
| 81 | HP | FISH+ | WT | WT | WT | WT |
| 82 | LP | FISH+ | WT | WT | E545K | WT |
| 83 | HP | FISH+ | WT | WT | WT | WT |
| 84 | D | FISH- | WT | WT | WT | WT |
| 85 | LP | FISH- | WT | WT | WT | WT |
| 86 | n.e. | n.e. | WT | WT | WT | WT |
| 87 | LP | FISH+ | WT | WT | WT | WT |
| 88 | LP | FISH- | WT | WT | WT | WT |
| 89 | HP | FISH+ | WT | WT | WT | WT |
| 90 | LP | FISH+ | WT | WT | WT | WT |
| 91 | HP | FISH+ | WT | WT | WT | WT |
| 92 | n.e. | n.e. | WT | WT | WT | WT |
| 93 | A | FISH+ | WT | WT | WT | WT |

Molecular profile of SCAC patients. A: amplification, D: disomy, FISH+: ≥4 copies of EGFR in ≥40% of cells or amplification, FISH-: ≥4 copies in <40% of cells, loss: loss of chromosome 7, HP: high polysomy, LP: low polysomy, n.e.: not evaluable, wt: wild-type.

## 4.5 CORRELATION BETWEEN CLINICAL PATHOLOGICAL AND MOLECULAR RESULTS

Analysis of correlation among clinical pathological features (age, sex, G and keratinization) revealed that patients age was associated with tumor G (both for patients older than 50 and older than 40 years) ($p< 0.01$ and $p= 0.04$, respectively). G was also associated with keratinization ($p< 0.01$). A trend of correlation was observed between G and patients sex ($p= 0.057$) (Table 9).

**Table 9**

| | Sex | Grade | Keratinization |
|---|---|---|---|
| Age (50 years) | 0.12 | **0.00007** | 0.74 |
| Age (40 years) | 0.66 | **0.04** | 1 |
| Sex | - | 0.057 | 1 |
| Grade | - | - | **0.000005** |

P values of clinical-pathological correlation by Fisher's Exact Test. Significant values in bold.

No significant correlation has been found between clinical pathological features and EGFR gene status (presence of A, D, FISH+ or FISH- profile) as detected by FISH and molecular alterations in KRAS and PIK3CA (both in exon 9 and in exon 20) (Table 10).

**Table 10**

| | mut KRAS | mut PIK3CA | mut PIK3CA ex9 | mut PIK3CA ex20 | EGFR* | EGFR** | FISH* | FISH** |
|---|---|---|---|---|---|---|---|---|
| Age (50 years) | 1 | 0.69 | 1 | 1 | 0.5 | 0.38 | 0.13 | 0.67 |
| Age (40 years) | 1 | 0.59 | 1 | 1 | 1 | 0.14 | 0.62 | 0.13 |
| Sex | 1 | 1 | 1 | 1 | 0.57 | 0.63 | 1 | 0.29 |
| Grade | 1 | 0.4 | 0.7 | 0.7 | 0.48 | 0.49 | 0.65 | 0.71 |
| Keratinization | 1 | 1 | 1 | 1 | 0.14 | 1 | 1 | 0.49 |
| KRAS | - | 1 | 1 | 1 | 1 | 0.64 | 0.15 | 0.48 |
| PIK3CA | - | - | - | - | 1 | 0.54 | 0.35 | 0.82 |
| PIK3CA ex9 | - | - | - | 1 | 1 | 0.74 | 0.74 | 0.92 |
| PIK3CA ex20 | - | - | - | - | 1 | 0.57 | 0.29 | 1 |

P values of molecular correlation by Fisher's Exact Test. EGFR*: A vs. not A, EGFR**: D vs. not D, FISH*: FISH+ vs. FISH-, FISH**: L vs. D vs. LP vs. HP vs. A.

# 5 DISCUSSION AND CONCLUSION

SCAC is a rare disease, representing 1.5% of all the gastrointestinal tumors (Martin FT. et al, 2009). HPV is detected in the majority of SCAC patients and HR-HPV infection is considered the initiating event in anal epithelial transformation (Gervaz P. et al, 2006).

Patients with primary SCAC are traditionally managed with chemoradiation, which results in complete response in up to 90% of cases. In non-responders or recurrent patients, salvage APR, that results in a permanent colostomy, is recommended (Gervaz P. et al, 2008; Czito BG. et al, 2009; Meyer J. et al, 2010). To overcome the severe side effects of this surgical procedure new therapeutic options are being evaluated. A very promising approach is represented by treatments able to inhibit molecules that are fundamental for tumor growth, the so called targeted therapies.

In the past few years, EGFR, a TK receptor that finely regulates cell proliferation and cell survival (through the RAS/RAF/MEK/ERK and PI3K/PTEN/AKT pathways), has gained increased importance due to its role as a target for tailored treatment. Recently the anti-EGFR monoclonal antibody cetuximab has been FDA and EMA approved as therapy for different tumors, both squamous and adeno-carcinomas, such as advanced or recurrent HNSCC and mCRC (Gazdar AF., 2010; Kendall A. et al, 2010; Tejani MA. et al, 2010).

Similarly, in order to overcome failure of conventional therapies and the consequences of the ablative surgery, the use of cetuximab has been

proposed in SCAC, on the basis of some molecular features of this cancer, such as EGFR protein overexpression and gene copy number gain, which have been described in a significant proportion of cases (Le LH. et al, 2005; Alvarez G. et al, 2006; Walker F. et al, 2009; Van Damme N. et al, 2010).

At the moment, only sporadic studies have investigated the use of cetuximab in SCAC, reporting activity in very few patients affected by a refractory or metastatic disease. Moreover, little is known in SCAC not only about EGFR gene copy number gain, that seems to be a predictive marker of cetuximab efficacy, but also about EGFR-downstream members alterations (KRAS, PIK3CA and BRAF mutations), which are known to be markers of resistance to anti-EGFR therapies in patients affected by mCRC (Moroni M. et al, 2005; Lievre A. et al, 2006; Benvenuti S. et al, 2007; Frattini M. et al, 2007).

Phan and colleagues, evidenced a female patient with refractory SCAC who achieved an excellent response to the combination of cetuximab and irinotecan after having failed single-agent irinotecan (Phan LK. et al, 2007). Lukan reported a disease control in 5 out of 7 patients with metastatic SCAC treated with cetuximab, in first or subsequent lines instead of cisplatin-based therapy, and interesting revealed that all the 5 responders patients were characterized by absence of mutations in KRAS gene, in contrast to both patients with progressive disease that showed KRAS mutations (Lukan N. et al, 2009). Our group described about one refractory SCAC patient that benefited from the same combination and that showed EGFR gene copy number gain and absence of mutations in KRAS gene (De Dosso S. et al, 2010). Finally, Saif described a good response after cetuximab or panitumumab in 3 cases of refractory SCAC (Saif M. et al, 2011).

These anecdotic findings reproduce those obtained in mCRC, thus supporting the importance of EGFR investigation, and confirming the role of KRAS as a putative predictive marker of non-response to cetuximab also in SCAC. Similarly, they suggest the possible role of other EGFR downstream members, such as PIK3CA and BRAF, in affecting the efficacy of anti-EGFR agents also in SCAC (Mao C. et al, 2009; Bardelli A. et al, 2010).

The only study investigating PIK3CA in SCAC identified 5 patients with mutations out of 127 patients (5%) (Patel H. et al, 2007).

BRAF mutations in SCAC have not been investigated yet, thus indicating the need of additional studies on this issue.

Therefore, we investigated EGFR and its downstream pathway in the same cohort of SCAC by analyzing EGFR gene status by FISH and KRAS, BRAF and PIK3CA mutations by sequencing.

In this study we analyzed 93 patients affected by SCAC.

In line with literature data, the great majority of them (96%) showed integration of the HPV virus, thus supporting the hypothesis that infection is one of the driving events in the tumorigenesis of this cancer.

More than thirty percent of the investigated patients were characterized by EGFR gene copy number gain (i.e. FISH+, 37%), confirming the very few published studies reporting a rate of polysomy ranging from 9% to 33% (Le LH. et al, 2005; Alvarez G. et al, 2006; Walker F. et al, 2009; Van Damme N. et al, 2010). Interestingly, we documented for the first time the presence of EGFR gene amplification in four SCAC patients. Of these, three patients showed high level of gene amplification with large clusters of signals in all the tissue, whereas one patient showed a low level of gene amplification in

different tumor areas, thus probably suggesting that different biological mechanisms might be involved in the activation of this oncogene in SCAC.

Concerning EGFR-downstream members, we detected KRAS gene mutations in four patients (corresponding to 4% of our cohort), confirming that this alteration may occur in a subgroup of SCAC, as reported by Lukan and coauthors (Lukan N. et al, 2009), and that is not a rare event, as indicated in two recent studies that did not find any KRAS mutation out of a total of 82 investigated SCAC patients (Zampino MG. et al, 2009; Van Damme N. et al, 2010).

Investigation in PIK3CA gene sequence identified a group of 13 mutated patients out of 89 (15%) analyzable SCAC. The majority of these (10/13=77%) presented a mutation in exon 9, whereas only 3 cases were mutated in exon 20. Distinction about the site of mutation in PIK3CA gene is extremely important. It has been recently demonstrated in mCRC that patients with mutation in exon 20 of PIK3CA are resistant to cetuximab treatment, whereas patients with mutation in exon 9 have the same likelihood of response to cetuximab that have patients with PIK3CA wild type gene (De Roock W. et al, 2010); therefore in our cohort we could enumerate only 3 patients having a PIK3CA mutation that is a putative predictive marker of resistance to EGFR-targeted therapies.

Our results about PIK3CA mutations showed some discrepancies when compared with data obtained in the lonely published study concerning PIK3CA mutation analyses in SCAC (Patel H. et al, 2007). Differences were related to the frequency of mutations and to the type of mutations (exon 9 vs. exon 20). In details, Patel and colleagues found PIK3CA mutations in 5 patients out of 127 SCAC, with a rate of mutation lower than what has been

obtained in our study (4% vs. 16%); also they found that the majority of mutations involved exon 20 (4 patients) rather than exon 9 (1 patient), the opposite of our results (10 patients with mutation in exon 9 and 3 patients in exon 20). Nevertheless, even if some epidemiological effects may play a role, our results are similar with general data concerning frequency of PIK3CA mutations in SCC at other sites, that are in a range of 6-10% (Kozaki K. et al, 2006; Mori R. et al, 2008; Murugan AK. et al, 2008; Akagi I. et al, 2009).

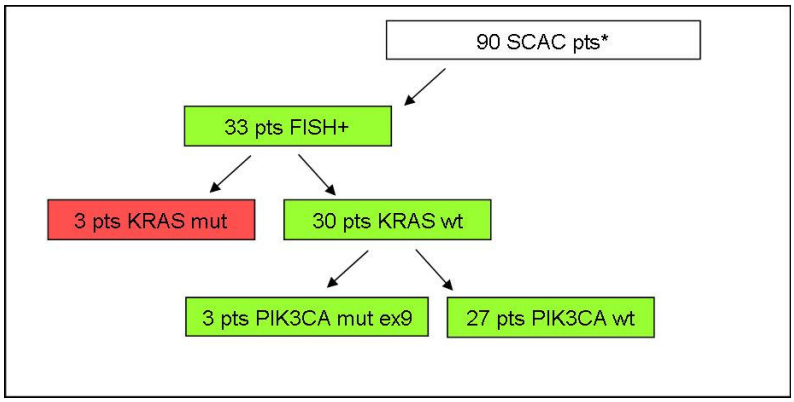
Finally, in this work we investigated for the first time the presence of BRAF gene mutation in SCAC patients. Our findings indicated absence of mutations in BRAF gene, similarly to other SCC, where it is reported as a rare event (<3% of patients), thus suggesting that probably this gene plays a minor role (if any) in SCAC (Cosmic, Catalogue of Somatic Mutation: http://www.sanger.ac.uk/genetics/CGP/cosmic/). Further studies investigating larger series of SCAC patients are needed to confirm and validate this hypothesis.

Overall, our results revealed that: i) chromosome 7 polysomy seems to be the principal mechanisms of EGFR deregulation, whereas gene amplification is rare, but not absent, as stated at the moment in literature; ii) KRAS and PIK3CA mutations may be identified in a subgroup of SCAC patients; iii) BRAF does not play a relevant role in SCAC tumorigenesis.

In conclusion, our study characterized in the same cohort of SCAC the deregulation of EGFR and of its downstream members. Assuming that evidences obtained from mCRC and HNSCC are valid for SCAC (hypothesis that is confirmed at the moment in some anecdotic SCAC treated with cetuximab) around 30% of SCAC patients have a proficient molecular profile to be addressed to EGFR-targeted therapies (Phan LK. et al, 2007; Lukan N.

et al, 2009; De Dosso S. et al, 2010; Saif M. et al, 2011). This subgroup is represented by SCAC patients whit EGFR gene copy number gain (FISH+), absence of mutation in KRAS, absence of mutation in PIK3CA or mutation in PIK3CA exon 9 (Figure 9). On these bases, it is strongly recommended that, in the future, prospective clinical trials should be proposed with evidences for these molecular markers.

**Figure 9**




Algorithm of patients selection based on cytogenetical and molecular profile.
In green SCAC patients who are likely to benefit from anti-EGFR targeted therapy; in red putative non responder SCAC patients.

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