No evidence of NRAS mutation in squamous cell anal carcinoma (SCAC)

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Epidermal growth factor receptor (EGFR) is usually expressed in squamous cell anal carcinoma (SCAC) and anti-EGFR agents could represent a valid treatment strategy, also considering that KRAS and BRAF mutations are rare events in this type of cancer. However, no data are available on NRAS status in SCAC. In this study we analyzed NRAS status (exons 2–4) by Pyrosequencing in a case series of 50 SCAC patients previously characterized in our laboratory for KRAS, BRAF, PIK3CA mutations and HPV and HIV infections. We found no mutation in NRAS gene. These results confirm that since the principal anti-EGFR resistance mechanisms are almost absent in SCAC, anti-EGFR agents should be considered for the treatment of this type of cancer.

Anal cancer accounts for about 1–5% of all gastrointestinal malignancies, and infection of human papilloma virus (HPV) represents the main etiologic factor\textsuperscript{1}. The standard treatment for locoregional disease is combined chemoradiation, which is associated with a 3-year disease-free survival of about 70%. Of those patients who relapse following chemoradiotherapy (CRT), around 40% undergo salvage therapy with abdominoperineal resection\textsuperscript{2}. Locally advanced or metastatic disease is still incurable, with only limited responses reported for palliative chemotherapy\textsuperscript{3}. New therapeutic approaches are required for these patients.

As EGFR expression in anal carcinoma is observed in a high number of patients (about 80–90% of cases)\textsuperscript{4–6}, anti-EGFR drugs have been studied for the treatment of SCAC, showing anti-tumoral activity\textsuperscript{7–10}. The use of anti-EGFR monoclonal antibodies (mAb anti-EGFRs) in colorectal cancer has shown that KRAS and NRAS represent the main resistance mechanisms to this type of treatment and are thus used in clinical practice to select the patients who should not be treated with anti-EGFR therapy\textsuperscript{11}. BRAF and PIK3CA could also have a prognostic role in determining those patients with a worse outcome\textsuperscript{12–14}. KRAS mutation seems to be a rare event in SCAC. Some studies, including a study from our group, have shown that no KRAS mutation is present in SCAC\textsuperscript{4–6,15,16}, whereas others have reported rates of KRAS mutation of 1–5%\textsuperscript{17,18}. A low frequency of BRAF mutations has also been observed in SCAC, with a rate of 0–5%\textsuperscript{15,17,18}. A higher rate of PIK3CA mutation between 16% and 22%, has been observed\textsuperscript{15,18}. However, no correlation with patient prognosis was observed for these mutations, as well as for HPV infection. To date, no data are available on the frequency of NRAS mutation in SCAC. In this study we analyzed the status of NRAS in a case series of SCAC previously characterized for HPV, KRAS, BRAF and PIK3CA.

Materials and Methods

Case Series. We analyzed a case series of 50 consecutive SCAC patients treated with chemotherapy and radiotherapy at Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS in Meldola and at the Medical Oncology Units of Faenza, Ravenna and Macerata Hospitals from March 2001 to August 2012, for whom histological material was available. Forty-eight patients received the Nigro scheme with standard dosages\textsuperscript{19}, while

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the remaining 2 patients underwent treatment with cisplatin plus 5-fluorouracil. This case series was previously characterized by our group for HPV infection, KRAS (exons 2–4), BRAF (exons 11 and 15) and PIK3CA (exons 9 and 20) status15. The study protocol was reviewed and approved by the Medical Scientific Committee of IRST IRCCS, and written informed consent was obtained from patients or from their next of kin for the use of biological samples for research purposes. In addition, the experiments in this study were conducted in accordance with approved guidelines and regulations.

**Mutation analysis.** Genomic DNA was extracted from Formalin-fixed paraffin-embedded (FFPE) tumor blocks, as previously described15. NRAS (exons 2, 3 and 4) status was analyzed by Pyrosequencing using anti-EGFR MoAb response (NRAS status) (Diatech, Jesi, Ancona, Italy), according to the manufacturer’s instructions. Reactions were run on a PyroMark Q96 ID (Qiagen, Hilden, Germany).

**Statistical analyses.** Descriptive statistics were reported as frequencies and percentages for categorical variables and median and range for continuous variables. Progression-free survival (PFS) was calculated from the first day of treatment to the date of first observation of disease progression or death resulting from any cause, whichever occurred first, or the last follow-up for patients who were still alive and had not progressed. Overall survival (OS) was calculated from the first day of treatment to the date of death from any cause or the last follow-up. PFS, OS and their 95% confidence intervals (95% CI) were estimated using the Kaplan-Meier life-table method and survival curves were compared by the logrank test. Statistical significance was assumed for P < 0.05. Statistical analyses were carried out with SAS Statistical software (version 9.4, SAS Institute, Cary, NC, USA).

**Results**

Clinical-pathological characteristics of SCAC patients included in this study are reported in Table 1. Fifty patients (19 males, 31 females) were analyzed. Median age was 62 years (range, 37–87 years). Twenty-nine (58%) patients had early stage disease (stage I and II), 19 (38%) had regional nodal involvement (stage III), and 2 (4%) had distant metastasis (stage IV). All patients were KRAS (exons 2–4) and BRAF (exons 11 and 15) wild-type (wt), whereas 11 showed a PIK3CA mutation (8 at exon 9 and 3 at exon 20). All patients had HPV infection (45 HPV type 16 and 5 other HPV types), 6 of which had also HIV infection. All 50 patients resulted wt for codons 12–13 (exon 2), 58–59–61 (exon 3) and 117–146 (exon 4) of NRAS gene.

At a median follow-up of 50 months (range 2 to 178 months), 11 patients had progressed (7 with local and 4 with distant relapse) and 10 had died from causes other than SCAC. PFS was 82% (95% CI 71–93) at one year, 67% (95% CI 53–82) at 3 years, and 52% (95% CI 34–70) at 5 years. OS was 93% (95% CI 86–100) at 1 year, 73% (95% CI 60–87) at 3 years, and 52% (95% CI 34–70) at 5 years. (Fig. 1 and Table 2). PFS and OS in relation to clinical-pathological characteristics of patients and in accordance to PIK3CA status, HIV and HPV infections are reported in Table 2. Tumor grade and size, lymph node involvement and stage were significantly associated with PFS and OS. Presence of HIV infection, type of HPV infection and PIK3CA status were not correlated with survival.

**Discussion**

The aim of our study was to analyze the status of NRAS gene in a case series of SCAC patients previously characterized in our laboratory for KRAS, BRAF and PIK3CA genes. To our knowledge, this is the first study to analyze NRAS status in SCAC. Given that mutations occurring on KRAS exon 2–4 in colorectal cancer – in addition to those occurring on NRAS exon 2–4 – may play a role in determining resistance to moAb-EGFRs11, these molecular characterizations could also be investigated in SCAC in view of the potential usefulness of these antibodies in this type of cancer.

Other studies have analyzed the frequency of KRAS, BRAF and PIK3CA mutations in SCAC, showing a very low rate of KRAS and BRAF mutations15–18, and a slightly higher frequency of PIK3CA mutation15,19. No correlation between PIK3CA mutation and patients’ prognosis was reported in this study with an updated follow up, confirming what previously stated15.

We demonstrated that no NRAS mutation is present in SCAC and confirmed the absence of potential resistance mechanisms to moAb-EGFR, such as cetuximab or panitumumab. This data, together with previous evidence on KRAS and BRAF, suggest that cetuximab could be a valid treatment strategy in SCAC.

We confirmed that PIK3CA mutations have no prognostic role in this type of cancer, whereas tumor grade and size, lymph node involvement and stage are significantly associated with PFS and OS. Moreover, infection of HPV 16 was significantly associated with a longer PFS but not with OS.

A recent phase I study has demonstrated that the adding of cetuximab to a chemo-radiotherapy with 5-fluorouracil (5-FU) and mitomycin-C in SCAC induced a complete remission rate of 73% with a relative high toxicity10. High toxicity was observed also in other clinical trials20,21, suggesting that the relation between toxicity, chemotherapy dosage and radiation techniques should be better clarified to optimize this promising combination treatment strategy for SCAC. In addition to this data, a recent case report has shown a dramatic response to cetuximab in combination with cisplatin and 5-FU in a patient with metastatic anal cancer2.

Moreover, there are no therapies available after progression to cisplatin and 5-FU that can improve survival. A case report showed a complete response to cetuximab in monochemotherapy in a patient with refractory metastatic anal carcinoma suggesting a possible use in this setting22.

There are numerous similarities between SCAC and head and neck squamous cell carcinoma (HNSCC), both in terms of etiology and pathology. In particular, as observed in SCAC, high EGFR expression and a low percentage of RAS mutations have also been reported in HNSCC23–25, and this was the basic starting point for research...
|                                | n (%) |
|--------------------------------|-------|
| **Gender**                     |       |
| Female                         | 31 (62) |
| Male                           | 19 (38) |
| **Age, years**                 |       |
| <50                            | 7 (14) |
| ≥50 and <70                    | 29 (58) |
| ≥70                            | 14 (28) |
| **Grade**                      |       |
| G1                             | 2 (4)  |
| G2                             | 32 (64) |
| G3                             | 16 (32) |
| **TNM (T)**                    |       |
| t1-2                           | 27 (54) |
| t3-4                           | 23 (46) |
| **Stage**                      |       |
| I                              | 8 (16)  |
| II                             | 21 (42) |
| III                            | 19 (38) |
| IV                             | 2 (4)  |
| **HIV infection**              |       |
| Yes                            | 6 (12)  |
| No                             | 44 (88) |
| **HPV infection**              |       |
| HPV-16                         | 45 (90) |
| Other HPV                      | 5 (10)  |
| **PIK3CA status**              |       |
| wt                             | 39 (78) |
| mutated                        | 11 (22) |
| **KRAS status**                |       |
| wt                             | 50 (100) |
| mutated                        | ---    |
| **BRAF status**                |       |
| wt                             | 50 (100) |
| mutated                        | ---    |

Table 1. Clinical-pathological characteristics of patients. HIV, human immunodeficiency virus; HPV, human papillomavirus. wt, wild type.

Figure 1. Kaplan-Meier survival curves of (A) progression-free survival (PFS) and (B) overall survival (OS).
into mAb anti-EGFRs in this type of cancer. The approval of cetuximab for the treatment of advanced and metastatic HNSCC26,27 further reinforces the potential effectiveness of anti-EGFR treatment in SCAC.

A limitation of the study consists in the fact that, due to its retrospective nature, not all information on the chemoradiotherapy administered was available, in particular that pertaining to the different radiotherapy dosages delivered. Moreover, despite the known disadvantages of a composite endpoint, we decided to use such an endpoint because of the potential underreporting of disease recurrences caused by the non-homogeneous availability of follow-up data from the different centers involved in the study.

Our results demonstrated that NRAS gene is wt in SCAC patients. These data, together with those reported on KRAS status, seem to confirm the potential usefulness of anti-EGFR drugs in the treatment of SCAC. Results of ongoing clinical trials will clarify the tolerability and the efficacy of these agents in combination with conventional CRT.

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**Table 2. PFS and OS in relation to clinical-pathological characteristics of patients.**

| Stage | n patients | % 1 year (95% CI) | % 3 years (95% CI) | % 5 years (95% CI) | P | % 1 year (95% CI) | % 3 years (95% CI) | % 5 years (95% CI) | P |
|-------|------------|------------------|------------------|------------------|---|------------------|------------------|------------------|---|
| Overall | 50 | 82 (71–93) | 67 (53–82) | 52 (34–70) | — | 93 (86–100) | 73 (60–87) | 52 (34–70) | — |
| Grade | | | | | | | | | |
| 1 + 2 | 34 | 87 (75–99) | 76 (60–92) | 55 (31–79) | — | 93 (85–100) | 78 (63–94) | 59 (36–82) | — |
| 3 | 16 | 73 (51–96) | 49 (22–76) | 39 (11–67) | 0.048 | 93 (81–100) | 63 (37–89) | 36 (8–63) | 0.040 |
| T | | | | | | | | | |
| 1–2 | 27 | 92 (81–100) | 87 (73–100) | 58 (32–84) | — | 96 (88–100) | 86 (72–100) | 62 (35–88) | — |
| 3–4 | 23 | 76 (55–97) | 46 (19–73) | 46 (19–73) | — | 88 (73–100) | 53 (26–79) | 44 (17–71) | — |
| Lymph node status | | | | | | | | | |
| Negative | 31 | 86 (73–99) | 78 (63–94) | 57 (35–79) | — | 100 | 85 (71–99) | 59 (37–81) | — |
| Positive | 19 | 76 (56–97) | 44 (17–72) | 44 (17–42) | 0.037 | 82 (63–100) | 51 (24–79) | 41 (13–69) | 0.013 |
| HIV status | | | | | | | | | |
| Negative | 44 | 80 (68–92) | 69 (55–84) | 53 (35–72) | — | 93 (85–100) | 74 (60–88) | 55 (37–73) | — |
| Positive | 6 | 100 | 33 (0–87) | 33 (0–87) | 0.521 | 100 | 67 (13–100) | 0 | 0.324 |
| PIK3CA status | | | | | | | | | |
| wt | 39 | 83 (71–96) | 68 (52–84) | 54 (34–73) | — | 92 (83–100) | 73 (57–88) | 56 (37–75) | — |
| mutated | 11 | 78 (51–100) | 65 (32–97) | 43 (2–84) | 0.883 | 100 | 75 (45–100) | 30 (0–75) | 0.860 |
| HPV | | | | | | | | | |
| 16 | 45 | 80 (68–92) | 66 (51–81) | 51 (32–71) | — | 93 (85–100) | 73 (58–87) | 48 (28–68) | — |
| #16 | 5 | 100 | 80 (45–100) | 53 (5–100) | 0.904 | 100 | 80 (45–100) | 80 (45–100) | 0.854 |
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Author Contributions
A.C.G. and P.U. designed the study, A.C.G., G.L.F., M.S., M.G., S.T., J.C. collected and analyzed patients clinical information, L.C. conduced the experiments, L.S. and M.P. were the pathologists that selected tumor tissues, E.S. performed the statistical analyses, L.C., A.C.G., P.U. wrote the manuscript. All authors reviewed the manuscript.

Additional Information
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