Gene amplification and protein overexpression of EGFR and ERBB2 in sinonasal squamous cell carcinoma

Running title: EGFR and ERBB2 in sinonasal squamous carcinoma

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Condensed abstract: Alterations in *EGFR* or *ERBB2* were observed in 46% of SNSCC, while mutations in KRAS were absent. These findings indicate that therapies targeting these molecules could be a promising addition to the therapeutical possibilities for these tumors.

ABSTRACT

Background: Sinonasal squamous cell carcinomas (SNSCC) are rare tumors with no etiological link to tobacco and alcohol, as opposed to other squamous cell carcinomas of the head and neck. Despite improvements in the field of surgery and radiotherapy, patients with these tumors still face a very unfavorable prognosis, partly due to its localization in a complex anatomical area, which is of special relevance for surgery and postoperative treatment. Therefore, there is a need for new therapeutic possibilities for this tumor type.

Methods: Gene copy numbers of epidermal growth factor receptor (*EGFR*) and erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) were analyzed by FISH and MLPA and protein expression was evaluated by immunohistochemistry in 54 specimens SNSCC and the results were correlated with clinico-pathological and follow-up data.

Results: *EGFR* gene copy number increase were observed in 20/45 (44%) cases, and 21/54 (39%) tumors showed EGFR protein overexpression. Eight of 38 tumors (21%) showed *ERBB2* copy number increase, and 4/54 (7%) exhibited elevated protein expression levels. Both copy number increase and protein overexpression of EGFR and ERBB2 were mutually exclusive. *KRAS* mutations were absent in 37 cases analyzed.

Conclusion: A substancial proportion of SNSCC carry alterations in *EGFR* or *ERBB2*. Together with the absence of *KRAS* mutations, these findings indicate that therapies targeting these molecules could be a promising addition to the therapeutical options for these tumors.

Key Words: maxillary sinus, ethmoid sinus, sinonasal, squamous cell carcinoma, EGFR, ERBB2, KRAS, therapy.

INTRODUCTION

Sinonasal squamous cell carcinomas (SNSCC) are malignant epithelial tumors originating in the respiratory mucosa of the nasal cavities and paranasal sinuses. This is a complex anatomic area, close to structures such the eyes and the brain, which is of special relevance for surgery and postoperative treatment, since mutilation and aesthetic deformities are difficult to avoid[1]. SNSCC tend to present at advanced stages, with a high frequency of local failure and a moderate to poor outcome. Current treatment approaches include surgery, which may be accompanied by radiotherapy and, in some cases chemotherapy[1-5]. However, despite improvements in the field of surgery and radiotherapy, patients with these tumors still face a very unfavorable prognosis, with 5-year survival rate around 40% and local recurrence as the main cause of death[1-3]. Therefore, new therapeutic approaches are needed to improve these figures and the development of new treatment strategies for SNSCC remains a challenge.

Although it is well-known that SNSCC are etiologically related to occupational exposure to wood, leather and other types of organic dust[6-8], little is known about the genetic alterations underlying the development of SNSCC development. Previous reports indicate that SNSCC have a number of genetic changes in common with head and neck squamous cell carcinomas (HNSCC), in spite of differences in etiology[9]. This may be important with regard to clinical decision making and possibilities for new therapies, such as those directed against epidermal growth factor receptor (*EGFR*) and erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) proteins, because an example could be taken from the more prevalent and far more studied HNSCC[10].

EGFR and ERBB2 are members of the erbB family of tyrosine kinase receptor proteins. Interaction with the ligand promotes malignant transformation, angiogenesis, decrease apoptosis, metastatic dissemination, and responsiveness to chemotherapy. A number of studies have demonstrated that EGFR plays an important role in the pathogenesis of HNSCC, where it is expressed in up to 90% of tumors[11-13] and it has been associated with poor survival and risk of relapse[14-15]. ERBB2 overexpression is less frequent (up to 40% of

HNSCC) and its prognostic significance has not been well defined. EGFR antagonists are currently available for the treatment of HNSCC and ERBB2 antagonist are being used in the treatment of carcinomas in other locations such as the breast carcinoma.

There are few studies on the role of EGFR and ERBB2 in SNSCC and they show variable degree of EGFR (38-50%)[16-17] and/or ERBB2 protein overexpression (22%)[17]. Amplification of the 7p12 region (18%), where the gene that encodes EGFR is located, and 17q21 (18%), the site of the *ERBB2* gene, has been reported by our group previously by microarray CGH analysis[9]. Furuta *et al* reported *EGFR* amplification in 5/49 (10%) cases by slot blot analysis[18]. Some studies have suggested that EGFR and ERBB2 expression may be prognostic markers in SNSCC[19], others however reported that *EGFR* and *ERBB2* gene copy number increase may not be useful as a molecular marker[18]. The identification of biological markers such as these is important and may help to select patients with sinonasal SNSCC that most likely will respond to agents targeting these receptors.

Considering the morphological and molecular similarities existing between HNSCC and SNSCC, the aim of this study was to assess the frequency of EGFR and ERBB2 expression by immunohistochemistry (IHC) and gene copy number status by fluorescence *in situ* hybridization (FISH) and multiplex ligand-dependent probe amplification (MLPA) in SNSCC. We also examined the relationship between *EGFR* and *ERBB2* gene copy number and expression status and clinico-pathological parameters.

MATERIAL AND METHODS

Tumor Specimens

Between November 1989 and October 2009, tissue samples of 54 primary SNSCC were obtained from previously untreated patients undergoing surgical resection of their tumor at the Departments of Otolaryngology, Hospital Universitario Central de Asturias and Hospital General Universitario Gregorio Marañón (Madrid). Patients with primary tumors in the nasal vestibule or septum were excluded because these tumors probably arise from the skin rather than from nasal respiratory mucosa. All patients enrolled in the study gave written informed

consent for the collection, storage and analysis of specimens, and the study had received prior approval from the ethical committee of our institutions.

Clinical Variables

Of 54 patients, 38 were male (70%) and 16 were female (30%). The mean age was 66 years (median 66 years and range, 47-91 years). Forty-three tumors were located in the maxillary sinus (80%) and 11 in the ethmoid sinus (20%). The series comprised 18 well-differentiated tumors (33%), 11 moderately differentiated tumors (20%) and 25 poorly differentiated tumors (46%). In accord with the T stage of the UICC classification[20], 6 tumors were T2 (11%), 16 tumors T3 (30%), 24 tumors T4a (44%), and 8 tumors T4b (15%). If classifying by stage the series consisted of 4 tumors in stage II (7%), 18 tumors in stage III (33%), 24 tumors in stage IVa (44%), and 8 tumors in stage IVb (15%). At the time of surgery 14 patients (26%) had lymph node metastasis and no patient had distant metastases. The periorbit was affected in 15 patients (28%) and another 7 patients (13%) had clear orbital invasion. Eight patients (15%) had intracranial involvement at diagnosis. Forty-two patients (78%) received radiotherapy after radical surgery. The median follow-up time was 16 months (3–211 months). All clinical and tumor characteristic are presented in table 1.

Tissue microarray (TMA)

Tissue microarray blocks were assembled from formalin-fixed, paraffin-embedded tissues as previously described[21]. Areas of interest rich in non-necrotic areas were identified on corresponding hematoxylin and eosin-stained sections and marked with 2 mm circles on the source paraffin block. The source block was cored and a 1 mm core transferred to the recipient master block using the Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Three cores from different areas of the same tissue block were arrayed for each case. Four different TMA blocks were constructed with a total of 162 tissue cores with a spacing of 1.5 mm, representing 54 SNSCC. Three micrometer sections were stained with

hematoxylin and eosin and reviewed by one pathologist to determine whether the samples represented the tumor.

FISH

FISH was performed using a directly labeled dual probe EGFR/centromere 7 and ERBB2/centromere 17 (Kreatech Diagnostics, Amsterdam, The Netherlands) following the manufacturer's recommendations. Four µm TMA sections were pretreated using a commercial slide preparation kit (DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's recommendations. Images were analyzed and captured using the Olympus BX-61 fluorescence microscope mounted with a SpectraCube system and FISHview software version 1.6.1 (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Interphase nuclei in the tumor with intact morphology were counted for green *EGFR* and red centromere 7 signals using appropriate fluorecence filters and a 60X objective.

Scoring was done in two different ways. First, copy number gain was defined when >30% of cells showed three or more signals and amplification when five or more signals. Second, copy number gain was considered when the ratio *EGFR*/centromere 7 or *ERBB2*/centromere 17 signals was between 1 and 2 and amplification when 2 or more[22]. The latter approach is commonly used in practical clinical assessment of gene copy number and generally reflects the average copy number of the cell population examined. Representative examples of FISH are presented in Figure 1.

DNA extraction

Tumor DNA was extracted from frozen tissue samples and from paraffin-embedded tissue samples using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s recommendations. MLPA on DNA from frozen tumor tissue was analyzed by comparing to normal reference DNA extracted from blood of healthy donors and MLPA on DNA from paraffin tumor tissue was analyzed by comparing to normal reference DNA obtained from paraffin blocks of normal tissue.

Special care was taken to obtain high quality DNA from the formaldehyde-fixed, paraffin-embedded tissues. DNA extracted from archival material can be partly degraded and cross-linked, the extent of which depends on the pH of the formaldehyde and the time of the fixation before paraffin embedding. To improve the quality of the isolated DNA, we have applied an elaborate extraction protocol especially for paraffin tissues, which includes thorough deparaffination with xylene, methanol washings to remove all traces of the xylene, and a 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet is dried and digested for 3 days in lysis buffer with high doses of proteinase K (final concentration 2 ug/uL, freshly added twice a day). With this protocol, most formaldehyde-fixed, paraffin-embedded tissue samples yielded DNA of relatively good quality, with A260/A280 values between 1,7 and 2.0 measured by Nanodrop (Thermo Scientific, Wilmington DE, USA) and lengths between 2000 and 20,000 bp, and these samples were used in MLPA analysis.

MLPA

The MLPA tecnique has been performed as described in detail previously[23]. using the p105 kit which contains 11 probes for *EGFR*. Each probe is composed of two parts that hybridize to adjacent target sequences in the DNA. After a ligation step and a PCR amplification, each probe gives rise to a product with a unique size between 130 and 480 bp. Briefly, 100 ng DNA was denatured at 98ºC for 5 min and hybridized with the MLPA probe mixture at 60ºC for 16 hours. Ligation of the two parts of each probe was preformed by a thermostable ligase. All probe ligation products have the same end sequences and were amplified by PCR using the same primer pair for 60ºC 1 min, 33 cycles of 95ºC 30 sec, 60ºC 30 sec and 72ºC 1 min, followed by 20 min at 72ºC and kept cold at 4ºC. The products were subsequently analysed on an ABI Prism 3100 sequencer and by GeneScan v3.7 software (Applied Biosystems, Warrington, UK). Fourteen control experiments using normal DNA from 7 different donors were used to calculate median reference values and the standard deviations for every probe. Each tumor sample was analysed at least twice. For every gene, the relative copy number

was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. We observed that a MLPA experiment analysing a new normal DNA resulted in relative copy numbers varying between 0.85 and 1.15, including the standard deviation, for each probe. Therefore we decided that relative copy number values, including the standard deviation, lower than 0.8 were interpreted as losses, higher than 1.2 as gains and 2.0 or higher as amplifications. Representative examples of *EGFR* and *ERBB2* gene amplification are presented in Figure 1.

Immunohistochemistry

Immunohistochemistry was performed with mouse monoclonal EGFR antibody (Clon 2-18C9, DAKO, Glostrup, Denmark) and HercepTest™ kit K5206 (DakoCytomation, Glostrup, Denmark) using an automatic staining workstation (Dako Autostainer, Dako Cytomation, Glostrup, Denmark) with the Envision system and diaminobenzidine chromogen as substrate. All procedures were performed according to the manufacturer´s instructions with the reagents supplied with the kit. EGFR immunostaining was considered positive when strong (+++) membranous, cytoplasmic or both staining was observed in at least 10% of tumor cells, whereas cases with 0 to ++ staining were regarded as negative. ERBB2 immunostaining was considered positive when any membranous staining (+/++/+++) was observed in at least 10% of tumor cells, whereas cases without staining (0) were regarded as negative. Only membrane ERBB2 immunostaining was considered positive. Control samples showed weak ERBB2 staining. Representative examples of immunohistochemical staining obtained with these antibodies are presented in Figure 1.

***KRAS* mutation analysis**

KRAS exon 2 (codons 12 and 13) was analyzed by direct sequencing using the following primers; *KRAS* Exon 2F: TACTGGTGGAGTATTTGATAGTG, and *KRAS* Exon 2R: CTGTATCAAAGAATGGTCCTG. Reactions were set up in standard conditions with a 5'-first step at 95 ºC followed by 30 cycles (30" 95 ºC, 45" 60 ºC, 30" 70 ºC). Products were purified

with High Pure PCR product purification kit (Roche) following manufacturer's instructions. Sequencing PCR was set up with kit Big Dye Terminator® v1.1 (Applied Biosystems) and analyzed using the ABI PRISM 3100 and 3730 Genetic Analyzer (Applied Biosystems, Foster City CA, USA). Sense and antisense sequencing was performed for confirmation.

Statistical Analysis

Possible correlations between genetic and clinical parameters were statistically analyzed by SPSS 12.0 software for Windows (SPSS Inc., Chicago, IL), using the Pearson chi-square test and Fischer's exact test. Kaplan–Meier analysis was performed for estimation of survival, comparing distributions of survival through the logarithmic range test (log-rank test). Values of $p < 0.05$ were considered significant.

RESULTS

Follow up

During the time of follow-up, 45 patients developed local recurrence (83%), 5 of which also developed a distant metastasis (9%). At the time of writing, a total of 9 patients remained disease-free. The overall 5-year survival was 15%, and 1 and 5-year disease-free survival was 22% and 6%, respectively. The main causes of death in our series were local recurrences and distant metastasis. However, 5 patients died during the postoperative period or due to intercurrent causes. We found no significant relation between the overall survival and clinical parameters such as localization, histologic differentiation, tumor classification, disruption of the orbit, intracranial invasion or affected lymph nodes at the time of diagnosis. Patients with advanced tumors (T3-T4) ($p=0.021$) and with disruption of the orbit ($p=0.001$ developed early recurrence after treatment.

EGFR, *ERBB2* and *KRAS* analysis

FISH analysis yielded interpretable information in 45 of 54 tumors for *EGFR* and in 38 of 54 tumors for *ERBB2*, while MLPA and *KRAS* analysis was successful in 37 cases.

FISH analysis detected 20 of 45 (44%) cases with copy number gain of *EGFR*, of which two cases with high level amplification. Evaluated as *EGFR*/centromere 7 ratio, eight cases showed a value between 1 and 2, and two cases of 2 or higher. MLPA analysis detected only 4 cases with copy number gain, 3 of which with amplification. *ERBB2* gain was found in 8 of 38 (21%) cases by FISH, four of which showing high level amplification. Evaluated as *ERBB2*/centromere 17 ratio, four cases showed a value between 1 and 2, and four cases of 2 or higher. MLPA analysis detected 6 cases with copy number gain of which 4 with amplification (Table 2). *EGFR* and *ERBB2* amplification never occurred together in the same tumor. Both for *EGFR* and *ERBB2* FISH analysis, some of the tumors represented in the TMA could not be analyzed, either because of lack of tumor representation in the TMA-section, or due to locally high fluoreresccence backgrounds.

Immunohistochemical analysis showed positivity at the cell membrane in 21 of 54 tumors (39%) for EGFR and in 4 of 54 cases (7%) for ERBB2 (Table 2). All cases with high level amplification of EGFR also demonstrated protein overexpression, but there was no correlation between protein overexpression and gene copy number level. Overexpression occurred in many cases showing a normal copy number level by either FISH or MLPA. ERBB2 protein overexpression was exclusively found in the cases carrying *ERBB2* gene amplification, but not in those with low level copy number gain ($p<0,001$). EGFR and ERBB2 overexpression was mutually exclusive, neither of the cases showed expression of both proteins simultaneously. In total 25 of 54 (46%) cases showed either EGFR or ERBB2 overexpression.

All thirty-seven cases analyzed for the presence *KRAS* mutations in codons 12 and 13 showed a wildtype sequence.

Correlation to clinico-pathological and follow up data

EGFR copy number gain or amplification did not correlate to any of the clinico-pathological data. EGFR overexpression was significantly more frequent in patients with positive lymph nodes at the time of diagnosis: 9/21 (43%) versus 5/33 (15%) in patients with no affected lymph nodes (Fisher Exact Chi2 p=0.027), and disruption of the orbit: 14/21 (67%) versus 8/33 24%) in cases without orbit disruption (Fisher Exact Chi2 p=0.002). No correlation was found with other clinico-pathological parameters, including localization, histological differentiation and distant metastasis. In addition, no significant difference was found in overall and disease-free survival between tumors with and without EGFR overexpression or amplification. The two samples with *EGFR* gene amplification belonged to patients with advanced- T stage tumors (T3 and T4a) arising from the ethmoid sinus. These patients received postoperative radiotherapy. The periorbit was affected in both cases but neither intracranial involvement nor distant metastases was observed.

ERBB2 copy number gain and overexpression was related to intracranial invasion: 3/4 (75%) cases versus 5/50 (10%) in cases with no intracranial invasion (Fisher Exact Chi2 p=0.008). The four tumors that showed *ERBB2* amplification and protein overexpression were all T4 stage, and three of these four cases had intracranial involvement but no distant metastases. The orbit was not affected in any of the tumors with *ERBB2* amplification and protein overexpression. No correlation was found with other clinico-pathological parameters, including localization, histological differentiation and distant metastasis. Finally, no significant difference was found in the overall and disease-free survival between tumors with and without *ERBB2* amplification or overexpression.

DISCUSSION

Although *EGFR* and *ERBB2* were indentified more than two decades ago, clinical interest in these genes has been heightened by the discovery of *EGFR* and *ERBB2* antagonists. EGFR is overexpressed in most epithelial malignancies, including colorectal and pancreatic

adenocarcinoma and HNSCC, and it has been correlated with an increased risk of locoregional tumor relapse following primary therapy and relative resistance to conventional treatment[24-27]. The role of ERBB2 expression in HNSCC is less defined in comparison to breast and ovarian cancer. Increased expression of ERBB2 has been shown in parallel with acquisition of a more malignant phenotype in a series of oral carcinomas, which may imply a role in progression[12,28].

Little is known about the involvement of EGFR and ERBB2 in SNSCC. A previous study of our group on a series of 29 cases of SNSCC indicated that the genetic profile is similar to HNSCC (despite etiological differences), including amplification of *EGFR* and *ERBB2*[9]. If this finding can be confirmed, then it may be possible to apply EGFR and ERBB2 antagonist therapy to SNSCC in the same way as HNSCC. The present study therefore aimed to analyze a larger series of cases for the presence of both gene copy number alterations and protein overexpression.

Our FISH results revealed that high *EGFR* gene copy number or amplification is not an uncommon event in SNSCC. The percentage of gene amplification is lower than in the study published by Furuta *et al*. who analyzed *EGFR* gene amplification in 49 cases of SNSCC and amplification was detected in 5 of the 49 cases (10%)[18]. As previously noted in different tumor types, the level of immunohistochemical expression does not exactly reflect the *EGFR* copy number status as determined by FISH analysis; we observed cases with normal gene copy number and positive EGFR expression and also cases with increased gene copy numbers and no EGFR expression. High level gene amplification as detected by FISH was always accompanied by protein overexpression. These values are lower than those observed in HNSCC, in which EGFR expression is found in up to 90% of tumors[11-13]. Franchi *et al.* measured EGFR in 55 patients with another sinonasal tumor type, intestinal-type adenocarcinomas, and approximately 32% of tumors showed EGFR expression[29].

ERBB2 gene copy number increase was less frequent than EGFR. Only the cases with *ERBB2* amplification patients had ERBB2 protein overexpressed, thus, unlike EGFR,

the level of protein expression exactly reflects the *ERBB2* gene copy number determined. This fact was previously noted in different tumors types such as breast and ovarian cancer[30]. Li *et al*. found a higher frequency of ERBB2 overexpression in 22% of carcinoma of the maxillary sinus in a group of 31 cases of SNSCC[17]. The finding of *ERBB2* amplification in SNSCC contrasts with published data in HNSCC in which no *ERBB2* amplification could be demonstrated[12]. On the other hand, the frequency of ERBB2 overexpression in SNSCC appeared very similar to HNSCC (both about 7%)[31].

Our data on SNSCC could not confirm the reported correlation between *EGFR* copy number amplification and protein overexpression and poor prognosis in HNSCC[14-15, 32-33]. However, we did find significantly more frequent overexpression of EGFR in patients with affected lymph nodes at the time of diagnosis and disruption of the orbit. These two clinical parameters are associated with poor prognosis in SNSCC[34]. Similarly, we could not confirm a relation between EGFR overexpression and recurrences, as reported by Miyaguchi *et al*[16]. It may be that the composition of our series of SNSCC, with mostly stages T3 or higher and with recurrrences in most cases, hampers a reliable follow-up and survival analysis. The relationship between EGFR overexpression and the development of metastatic lymph nodes may help select patients for prophylactic treatment of nodal areas potentially affected. However, our data do not lend support to this possibility and there is no consensus among authors regarding the need for prophylactic neck dissections in patients with SNSCC[2,35].

The role of ERBB2 in the pathogenesis of SNSCC is not clear, although its expression in healthy nasal mucosa has been demonstrated[36]. In the present study *ERBB2* amplification and overexpression was associated with advanced tumor stages (T4) and intracranial involvement, both being indicators of poor prognosis in SNSCC[34]. In addition, all four cases with *ERBB2* amplification and overexpression developed early recurrent disease and died of disease. Therefore, amplification/overexpression of *ERBB2* would prove to be an indicator of poor prognosis factor in SNSCC, similar to HNSCC[13,31]. We did not find an association with orbital involvement and distant metastases. The latter differs from the

published data in breast carcinomas where *ERBB2* amplification/overexpression correlates with the development of hematogenous metastasis and poor prognosis[37].

Since EGFR and ERBB2 protein overexpression was mutually exclusive, we grouped the positive cases together (21 cases with EGFR and 4 with ERBB2) and looked again for correlations to clinico-pathological features, however we found no new or stronger associations.

The frequencies of EGFR and ERBB2 alterations observed in SNSCC, although lower than those observed in HNSCC, colorectal adenocarcinoma, lung or breast cancer, indicate that they may play an role in a subset of SNSCC. Therefore we propose that there is a basis for investigating targeted therapies in this group of tumors. EGFR and ERBB2 directed therapy could be used in clinical practice by identifying and selecting those patients most likely to benefit from EGFR and ERBB2 inhibition, and this can be evaluated by FISH and immunohistochemistry.

In HNSCC, clinical trials are being performed with the EGFR antagonists cetuximab which appears to improve survival of patients with locally advanced or metastatic[38-40]. Small molecule tyrosine kinase inhibitors such as erlotinib, gefitinib or the dual EGFR/ERBB2 inhibitor lapatinib have shown only modest response rates[40]. In addition, recent studies have linked EGFR overexpression with resistance to chemo- and radiotherapy[41-42]. These HNSCC patients would be candidates for adjuvant treatment with selective inhibitors of EGFR. *In vitro* studies have indicated that treatment combining gefitinib with the ERBB2-targeting drug trastuzumab can improve therapeutic efficacy in patients with breast cancer and in HNSCC[43]. Moreover, it has been suggested that ERBB2 may contribute to resistance to anti-EGFR treatment and may have potential as predictive markers and as therapeutic targets for combination therapy in treatment of HNSCC[44]. A fortunate characteristic of SNSCC is the absence of *KRAS* mutations, similar to HNSCC[45-46] but opposed to colorectal adenocarcinomas, where it was shown that patients with *KRAS* mutations do not benefit from anti-EGFR monoclonal antibodies[47].

In conclusion, we have observed that EGFR and ERBB2 alterations are present in a substantial subset of SNSCC patients. In addition, *KRAS* mutations are absent. SNSCC share these characteristics with HNSCC. This is relevant with regard to clinical decision making and possibilities for new target therapies against EGFR and ERBB2, alone or in combination or as adjuvant treatment to radiotherapy.

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FIGURE LEGENDS

Figure 1A

DNA copy number and protein expression analysis of EGFR. FISH analysis on a 4 um paraffin section of a TMA using a dual *EGFR*/centromere 7 probe, labeled with PlatinumBright550 (red) and PlatinumBright495 (green), respectively. Most of the cells show three green centromeric and five or more red *EGFR* signals, indicating chromosome 7 aneuploidy and *EGFR* gene amplification (left panel). MLPA result of the same case, showing all 11 probes recognizing different exons of *EGFR* with a relative copy number of more than 1.5 (middle panel). Immunohistochemistry of the same case showing EGFR protein overexpression detected in the cytoplasm and the cell membrane (right panel). Original magnification 600x (left panel) and 200x (right panel).

Figure 1B

DNA copy number and protein expression analysis of ERBB2. FISH analysis on a 4 um paraffin section of a TMA using a dual *ERBB2*/centromere 17 probe, labeled with PlatinumBright550 (red) and PlatinumBright495 (green), respectively. Most of the cells show three or four green centromeric and five or more red *ERBB2* signals, indicating chromosome 17 aneuploidy and *ERBB2* gene amplification (Left panel). MLPA result of the same case, showing the 2 probes recognizing different exons of *ERBB2* with a relative copy number of more than 2.0 (middle panel). Immunohistochemistry of the same case showing ERBB2 protein overexpression detected in the cytoplasm and the cell membrane (right panel). Original magnification 600x (left panel) and 200x (right panel).

Table 1. Patient and tumor characteristics.

	Number of patients (%)
Gender	
Female	16 (30)
Male	38 (70)
Tumor site	
Maxillary sinus	43 (80)
Ethmoid sinus	11 (20)
pT classification	
T2	6 (11)
T3	16 (30)
T4a	24 (44)
T4b	8 (15)
Disease stage	
II	4 (7)
III	18 (33)
IVa	24 (44)
IVb	8 (15)
pN classification	
N0	40 (74)
N1	14 (26)
Histologic differentiation	
Well-differentiated	18 (33)
Moderately differentiated	11 (20)
Poorly differentiated	25 (46)
Orbit invasion	
No	32 (59)
Periorbit	15 (28)
Orbit	7 (13)
Intracranial invasion	
No	46 (85)
Yes	8 (15)

Table 2. Genetic and immunohistochemical results.

Gene	Copy number gain (%)		Protein overexpression (%)
	FISH	MLPA	
EGFR	20/45 (44%) 2 cases amplification	4/37 (11%) 3 cases amplification	21/54 (39%)
HER2	8/38 (21%) 4 cases amplification	6/37 (16%) 4 cases amplification	4/54 (7%)