Genetic Abnormalities Associated with Chemoradiation Resistance of Head and Neck Squamous Cell Carcinoma

Guido B. van den Broek,1,3 Volkert B. Wreesmann,1,3 Michiel W.M. van den Brekel,1,3 Coen R.N. Rasch,2 Alfons J.M. Balm,1,3 and Pulivarthi H. Rao4

Abstract

Purpose: To identify reliable predictors of chemoradiation resistance of advanced head and neck squamous cell carcinoma (HNSCC).

Experimental Design: We did a matched-pair analysis of 20 chemoradiation-resistant and 20 sensitive HNSCCs, identified among a series of 104 consecutively treated cases. We compared the global DNA copy number profiles derived from comparative genomic hybridization analysis of both groups to identify genetic markers associated with chemoradiation resistance.

Results: Although sensitive and resistant case groups were characterized by a similar total number of genetic aberrations, high-level amplifications were more frequent in resistant tumors. Resistant tumors were characterized by a different profile of genetic changes. Gains of 3q11-q13, 3q21-q26.1, and 6q22-q27 and losses of 3p11-pter and 4p11-pter were significantly associated with chemoradiation resistance. High-level amplifications unique to resistant cases involved the chromosomal regions 1p32, 3q21-q26.1, and 6q22-q27 and losses of 3p11-pter and 4p11-pter were significantly associated with chemoradiation resistance. High-level amplifications unique to resistant cases involved the chromosomal regions 1p32, 3q21-q26.1, and 6q22-q27 and losses of 3p11-pter and 4p11-pter were significantly associated with chemoradiation resistance.

Conclusions: Sensitive and resistant HNSCCs are characterized by divergent genomic profiles. These profiles may be valuable as predictive markers of treatment failure.

Head and neck squamous cell carcinoma (HNSCC) is an aggressive disease with a large proportion of cases presenting with advanced (stage III/IV) disease (1). Adequate management of advanced HNSCC requires an aggressive approach but is limited by the density of vital and functionally important structures in the head and neck region (2). In recent years, the application of concurrent platinum-based chemoradiation has emerged as an attractive alternative to traditional surgical management of advanced HNSCC. For example, primary chemoradiation offers the potential for functional preservation without survival compromise in the setting of advanced laryngeal carcinoma (3) and other advanced head and neck cancers. The significance of concurrent chemoradiation is further exemplified by recent studies showing survival benefit of concomitant chemoradiation over radiation therapy alone in the adjuvant setting or in case of unresectable HNSCC (4–8).

Although these studies clearly show the overall sensitivity of HNSCC to concurrent chemoradiation, a significant number of individual cases experience locoregional treatment failure. These patients suffer potential side effects and toxicities of chemoradiation (mucositis, xerostomia, swallowing problems, ototoxicity, renal toxicity, and other toxicities) without benefit (9, 10). Clarification of the molecular basis of chemoradiation resistance is needed to reveal reliable predictors of treatment failure and provide clues for the development of novel therapeutic approaches aimed at modulation of chemoradiation resistance.

Resistance of tumor cells to radiation and cisplatin is likely multifactorial. Recent studies have shown that abrogation of proapoptotic p53 signaling may aid cellular survival after cytotoxic stress (11). Accordingly, chemoradiation-resistant HNSCCs show a high rate of p53 aberrations and increased expression of MDM2, a protein that shuttles p53 into degradative pathways (12, 13). In addition to p53 abrogation, several chromosomal alterations including amplifications of genes involved in detoxification of cytotoxic agents, deletion of genes involved in DNA repair, and various uncharacterized chromosomal alterations have been associated with chemotheray and radiation therapy resistance of malignancies such as HNSCC (14). For example, Akervall et al. (15) found a higher rate of chromosomal alterations in cisplatin-resistant HNSCC cell lines relative to their sensitive counterparts. These data suggest that chromosomal instability and associated selection for chromosomal alterations may underlie adaptability of HNSCC to selection pressures such as chemoradiation treatment.

To explore the suggested chromosomal basis for chemoradiation resistance further, we did a matched-pair comparative genomic
hybridization (CGH) analysis of chemoradiation-sensitive and chemoradiation-resistant HNSCCs. Our data suggest that chemoradiation-resistant and chemoradiation-sensitive HNSCCs are characterized by divergent chromosomal profiles, the significance of which remains to be determined.

Patients and Methods

Patient population and tissue samples. One hundred and four previously untreated consecutive patients with advanced (stage III/IV) HNSCC (oral cavity, oropharynx, supraglottic larynx, and hypopharynx) treated with the RADPLAT protocol (16) at The Netherlands Cancer Institute were the subjects of this study. Treatment consisted of four consecutive weekly selective intra-arterial infusions of cisplatin (150 mg/m²) simultaneous with i.v. sodium thiosulfate rescue combined with conventional radiotherapy (70 Gy) as described in detail elsewhere (17). Treatment response was evaluated 6 to 8 weeks after completion of radiotherapy by magnetic resonance imaging followed by examination under general anesthesia. Thereafter, patients were subjected to regular outpatient follow-up.

Twenty-six (25%) patients with histopathologically proven residual disease or recurrence after treatment were observed in the study group and deemed chemoradiation resistant. These were clinically matched with 26 patients without residual disease or recurrence after at least 2 years of follow-up (chemoradiation sensitive). Matching criteria included tumor volume, tumor-node-metastasis stage, age, gender, anatomic location, and infusion mode (unilateral or bilateral). Archival paraffin-embedded pretreatment biopsies from chemoradiation-sensitive and chemoradiation-resistant primary tumors were histologically confirmed to contain >70% of tumor tissue. DNA was extracted as described previously (18).

CGH. CGH analysis was done as described previously (19). Briefly, equal amounts (2 μg) of tumor DNA and normal human placenta DNA were labeled with fluorescein-12-dUTP (HTC) and Texas red-5-dUTP (Perkin-Elmer), respectively, coprecipitated with 15 μg of human cot-1 DNA (Invitrogen), and suspended in a hybridization mix (50% formamide/15% dextran sulfate/2× SSC). The suspension was hybridized for 2 days at 37°C onto metaphase chromosome spreads. On completion of hybridization, the slides were washed, and the chromosomes were counterstained with 4',6-diamidino-2-phenylindole to allow for their identification. Image analysis was done in the following way: 10 individual metaphases were captured for each case with a cooled charged coupled device camera attached to a Nikon Microphot-SA microscope and processed by Quantitative Imaging Processing System (Applied Imaging). The chromosomes were identified by 4',6-diamidino-2-phenylindole banding analysis and segmented; the local background was subtracted, and the median axis was identified. Red, green, and blue fluorescence was analyzed for all metaphase spreads, normalized to a standard length, and statistically combined to show the red/green signal ratio and 95% confidence intervals for the entire chromosome. Copy number changes were detected based on the variance of the red/green ratio profile from the standard of 1. Ratio values of 1.2 and 2.0 were defined as thresholds for gains and high-level amplifications, respectively. Losses were identified as ratios of 0.8 or less.

Statistical analysis. The relative risk of local relapse associated with the individual chromosomal alterations was estimated by comparison of the case with that of the matched controls, by conditional logistic regression methods for individually matched case-control studies (20). Differences in high-level amplifications were calculated with help of the χ² and McNemar tests. Relative risk estimates, two-sided P values, and 95% confidence intervals were calculated using SPSS 12.0.1. Due to the small number of cases, multivariable analysis was not done.

Results

Characteristics of study population. Six chemoradiation-resistant tumors could not be used for CGH analysis because of insufficient DNA quantity (n = 4) and poor quality of paraffin DNA, manifest as poor quality of hybridization images (n = 2). Consequently, the CGH data of 20 chemoradiation-resistant tumors and 20 chemoradiation-sensitive tumors (matched controls) were analyzed. Patients' characteristics are detailed in Table 1.

Comparison of chromosomal instability in chemoradiation-sensitive and chemoradiation-resistant cases. Chromosomal alterations were detected in all 40 tumors. Chemoradiation-resistant and chemoradiation-sensitive tumors did not differ significantly in the total number of detected chromosomal alterations (227 versus 231), the number of detected chromosomal gains (82 versus 71), or the number of detected chromosomal deletions (129 versus 152). However, chemoradiation-resistant cases were more often characterized by the presence of high-level amplifications compared with their chemoradiation-sensitive counterparts (10 versus 5; P = 0.01; Table 2). In addition, the total number of detected high-level

<table>
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Abbreviation: TNM, tumor-node-metastasis.

Table 2. High-level amplifications in sensitive (n = 20) and resistant (n = 20) tumors

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*P = 0.01, χ²

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amplifications was higher in chemoradiation-resistant cases (16 versus 8; P value is not significant; Table 2).

**Comparison of chromosomal profiles of chemoradiation-resistant and chemoradiation-sensitive cases.** A comparison of individual chromosomal alterations detected in chemoradiation-resistant and chemoradiation-sensitive cases is shown in Fig. 1. Chromosomal aberrations differentiating chemoradiation-resistant and chemoradiation-sensitive cases included gains of 5q11-q12, 6q23-q27, 8p21-p23, 10q11-q22, 15q13-q26, 18q21-q23, and 22 and losses of 2p22-p2p5, 5p11-pter, and 7q11-q22 (present in sensitive cases) and gains of 6p11-pter, 9, and Xq11-pter and loss of 18p11-pter (present in resistant cases). Statistical analysis of frequency distributions of individual chromosomal alterations revealed that gains of 3q11-q13 (P = 0.017), 3q21-q26.1 (P = 0.038), and 6q22-q27 (P = 0.036) and losses of 3p11-pter (P = 0.016) and 4p11-pter (P < 0.001) were significantly more common in chemoradiation-resistant HNSCC (Table 3). In addition, further analysis revealed that chemoradiation-resistant HNSCC (amplifications of 1p32, 3q24, 7p11.1, 7p11.2-12, 8p11.1, 8p11.1-12, 12q15, 13q21, 15q12, 18p11.3, and 18q11) and chemoradiation-sensitive cases (1p33-34, 2q31, 7q21, 7q22-31, 11q12, 12q11, and 14q13) were characterized by a completely different profile of high-level amplifications (Fig. 2; Table 2).

**Discussion**

The potential of human cancer cells to adapt to environmental selection pressures, such as chemotherapy and radiotherapy, is a major determinant of clinical treatment failure and associated survival reduction. In recent years, several molecular pathways have been identified that may mediate cellular responses to cytotoxic stress. In some instances, cancer cells may alter these pathways in their favor. Nonetheless, our understanding of resistance to cytotoxic agents is far from complete.

In the present study, we report a genome-wide exploration of chemoradiation resistance. Our data suggest that chemoradiation-sensitive and chemoradiation-resistant HNSCCs do not...
differ in the overall number of chromosomal alterations present in the genome of tumor cells. In the literature, the relationship between treatment resistance and chromosomal damage is conflicting. Some previous studies have suggested that tumors resistant to chemotherapy or radiotherapy are characterized by a higher number of chromosomal alterations. For example, Akervall et al. (15) found a higher number of chromosomal alterations in cisplatin-resistant HNSCC cell lines compared with their sensitive counterparts. These studies suggest that development of chromosomal instability facilitates adaptation of tumor cells to cytotoxic agents. In contrast, a significant number of studies report a lower overall number of genetic alterations in chemotherapy-resistant tumors, suggesting that chromosomal instability makes tumor cells more vulnerable to cytotoxic agents (21). Although it is difficult to compare our concomitant chemoradiation data with genetic data of patients treated on separate chemotherapy or radiation protocols, all cases were characterized by multiple chromosomal alterations, possibly influenced by their uniformly advanced tumor stage (22, 23). Therefore, we believe that our data do not contribute to an increased understanding of the suspected link between chromosomal instability and cellular adaptability to cytotoxic stress.

In addition to a possible relationship between the overall number of genetic changes and chemotherapy or radiation therapy response, several studies have suggested that resistance to these treatments may be mediated by a higher number of gene amplifications in treatment-resistant tumors. Rao et al. (19) found several high-level amplifications in germ cell tumors, which were restricted to cisplatin-resistant tumors only. Wang et al. (14) reported that genetic amplification of thymidylate synthase defines a subgroup of colorectal cancers resistant to 5-fluorouracil. In agreement with these findings, we observed that high-level amplifications are more common in chemoradiation-resistant tumors. This study showed that not only the number of patients with high-level amplifications (10 versus 5) was higher in the resistant group compared with the sensitive group but also the number of high-level amplifications (16 versus 8; Table 2). The exact meaning of these findings in the context of chemoradiation resistance will depend on the identification of the genes driving selection for these high-level amplifications and their functional annotation.

In addition to the overall number of chromosomal alterations, our study identified several specific chromosomal alterations that may be associated with response to chemoradiation treatment, including gains of 3q11-q13, 3q21-q26.1, and 6q22-q27 and losses of 3p11-pter and 4p11-pter that were significantly more common in chemoradiation-resistant tumors than in chemoradiation-sensitive tumors. The presence of squamous cell carcinoma–related oncogene in the 3q26 region differ in the overall number of chromosomal alterations present in the genome of tumor cells. In the literature, the relationship between treatment resistance and chromosomal damage is conflicting. Some previous studies have suggested that tumors resistant to chemotherapy or radiotherapy are characterized by a higher number of chromosomal alterations. For example, Akervall et al. (15) found a higher number of chromosomal alterations in cisplatin-resistant HNSCC cell lines compared with their sensitive counterparts. These studies suggest that development of chromosomal instability facilitates adaptation of tumor cells to cytotoxic agents. In contrast, a significant number of studies report a lower overall number of genetic alterations in chemotherapy-resistant tumors, suggesting that chromosomal instability makes tumor cells more vulnerable to cytotoxic agents (21). Although it is difficult to compare our concomitant chemoradiation data with genetic data of patients treated on separate chemotherapy or radiation protocols, all cases were characterized by multiple chromosomal alterations, possibly influenced by their uniformly advanced tumor stage (22, 23). Therefore, we believe that our data do not contribute to an increased understanding of the suspected link between chromosomal instability and cellular adaptability to cytotoxic stress.

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may be an explanation for the observed relationship between 3q21-26 overrepresentation and chemoradiation resistance (24). Squamous cell carcinoma–related oncogene drives selection for 3q26 overrepresentation in squamous cell carcinomas and is a key activator of Hedgehog signaling, which has been associated with chemoradiation resistance of squamous cell carcinomas (25, 26). In addition to 3q loss, loss of 3p has been linked to cytotoxic treatment resistance previously. Akervall et al. (15) reported loss of 3p associated with cisplatin-resistant HNSCC cell lines. In addition, Ogawa et al. (27) observed that loss of heterozygosity at 3p21 differentiated radiation-resistant from radiation-sensitive laryngeal cancers and was inversely related to larynx preservation. The 3p21.3 region harbors various genes (e.g., FUS1, RASSF1A, 101F6, and NPRL2; ref. 28), which play a role in cell proliferation, cell cycle kinetics, signaling transduction, ion transportation and exchange, apoptosis, and cell death. These genes, when deleted, may well modify chemoradiation sensitivity. In contrast to loss of 3p, no prior studies have directly associated gain of 6q or loss of 4p with cytotoxic treatment resistance of HNSCC. Although the 4p and 6q region are commonly altered in squamous cell carcinomas, no candidate genes have been described.

Several limitations from this study should be addressed. First, our results are based on a relatively small number of cases and matched controls. Based on this shortcoming and the significant number of matching criteria, it was not possible to match cases and controls perfectly. Although we did not find a statistically significant difference between the case and control groups in individual matching criteria, the possibility of a significant difference in the overall profile or influence by other, not included factors, remains an item of concern. For example, a possible difference in human papillomavirus positivity between cases and controls could in principle account for the detected genetic differences (29). This possibility is limited given the equal distribution of tonsillar carcinomas over the two groups. However, we assessed the frequency distribution of p16 immunopositivity, an established marker of human papillomavirus positivity. This analysis did not reveal evidence for human papillomavirus bias ($p = 0.21$, $\chi^2$; see Table 4). Therefore, we have no indication that the case and control groups differ significantly in individual clinicopathologic factors or their collective clinicopathologic profile, but the possibility remains difficult to exclude entirely. In addition to issues of matching, the examination of pretreatment biopsies harbors a potential limitation. It is conceivable that genetic aberrations that cause chemoradiation resistance develop during treatment, and a study comparing the genomic content of pretreatment and posttreatment biopsies might address this issue better. Given the study limitations, we believe that external validation of our data is warranted. We are currently in the process of generating a tissue microarray of all patients included in the chemoradiation trial at The Netherlands Cancer Institute. This will allow for fluorescent in situ hybridization experiments and immunohistochemistry from a series of independent patients with known clinical outcome to validate the identified genetic factors as prognostic markers of chemoradiation outcome. Thereafter, identification of candidate genes and analysis of their function is needed to help explain the role of chromosomal alterations in chemoradiation resistance.

In summary, we conclude that different genetic abnormalities can be identified in chemoradiation-resistant and chemoradiation-sensitive tumors. Identification of amplified/overexpressed genes at these sites may elucidate new genetic pathways of chemoradiation resistance in HNSCC.

### Table 4. p16 overexpression in sensitive (n = 20) and resistant (n = 20) tumors

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References

18. Isola J, De Vries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival par...
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