Featured Article

Genetic and Expression Profiles of Squamous Cell Carcinoma of the Head and Neck Correlate with Cisplatin Sensitivity and Resistance in Cell Lines and Patients

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ABSTRACT

Purpose: The choice of treatment for squamous cell carcinoma of the head and neck (SCCHN) is still primarily based on the tumor-node-metastasis classification. However, it is reasonable to believe that biological profiles of SCCHN may be independently associated with response to therapy. The aim of the present study was to examine genetic changes and gene expression profiles that might correlate with sensitivity to cisplatin [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] in 10 SCCHN cell lines.

Experimental Design: Five cisplatin-sensitive and five cisplatin-resistant cell lines [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] were studied by comparative genomic hybridization, spectral karyotyping, and cDNA microarray analysis (21,632 sequence-validated human cDNA; confirmation by reverse transcriptase-PCR for selected genes). For the MET proto-oncogene, which showed low expression in the chemosensitive cell lines, we did immunohistochemical staining on SCCHN of 29 patients who received induction chemotherapy.

Results: The five cisplatin-resistant cell lines showed significantly more genetic imbalances (regions of loss and amplification) and chromosomal abnormalities by comparative genomic hybridization and spectral karyotyping, respectively, than did the five cisplatin-sensitive cell lines. Microarray studies identified ~60 genes that clearly distinguish between the two groups of cell lines. Some of these genes are known to be involved in tumor progression, metastasis, and drug resistance. We identified low expression of c-met (immunohistochemistry) as a predictive factor for complete response in nondiploid tumors (P = 0.026).

Conclusions: We conclude that cisplatin sensitivity and resistance are related to distinctive differences in the genetic and expression profiles in individual SCCHN tumor cell lines and in SCCHN patients. The genes we have identified may serve as potential targets for novel treatment strategies.

INTRODUCTION

When induction chemotherapy, with cisplatin and 5-fluorouracil, was introduced for treatment of squamous cell carcinoma of the head and neck (SCCHN) in the early 1980s, complete response rates of 30 to 40% were initially reported (1, 2). Additionally, a higher frequency of organ preservation in laryngeal cancer patients was shown among those who responded to induction chemotherapy (3). However, no survival benefit has been associated with induction chemotherapy (4), which in part may be due to the time delay in initiating effective treatment among nonresponders (5). New treatment strategies involving concomitant chemotherapy and radiation therapy show promise of an improved survival benefit (8%) relative to conventional treatment, i.e., surgery and radiation therapy in combination or as single modality treatment in SCCHN (4).

Treatment strategies for SCCHN are still primarily based on tumor-node-metastasis classification. Biological markers that can predict the response to therapy have thus far not been introduced. It is reasonable to believe that biological features of the tumors play a key role in the clinical response to any given therapy. Optimizing treatment protocols by objective measurable methods according to the genetic and biological properties of individual tumors could eventually lead to a survival benefit and lower morbidity. A variety of prognostic biological markers correlated to survival have been described over the years, but very few of these have been tested for...
prognostic accuracy. Promising preliminary results have shown a correlation between low bcl-xL expression (6), as well as p53 overexpression, and tumor response to chemotherapy in pretreatment biopsy specimens from patients enrolled in a clinical trial (7). Additionally, conflicting results have been reported regarding TP53 mutations correlated with sensitivity (8, 9), as well as with resistance to cisplatin (10). Recently, we presented data supporting the hypothesis that the cell cycle-regulating gene CCND1 is a potential marker of response to cisplatin because overexpression of cyclin D1 correlated with favorable response to the drug (11). However, because the etiology of solid tumors is multifactorial, it would most likely be beneficial to identify a panel of biomarkers that reflect the biological features of the tumor, including chemosensitivity.

Recent reports by van’t Veer et al. (12) in breast cancer, Singh et al. (13) in prostate, Nielsen et al. (14) in soft tissue sarcomas, and Takahashi et al. (15) in renal cancer have shown the power of high-throughput microarray technology, which can generate the molecular signatures that can objectively discriminate between groups of tumors with different clinical outcomes.

The aims of the present study were to compare the cisplatin-sensitive and cisplatin-resistant cell lines by (a) their genetic features evaluated by comparative genomic hybridization and spectral karyotyping and (b) their gene expression profiles by cDNA microarray, which are additionally confirmed for primary tumors by immunohistochemical staining. Despite the clinical heterogeneity of SCCHN, we believe that gene expression will reveal the consistent and specific molecular changes that determine prognosis and treatment response.

**MATERIALS AND METHODS**

**Cell Lines.** Ten squamous cell carcinoma cell lines (Table 1) established at the University of Michigan (UMSCC) were cultured in a humidified atmosphere of 5% CO₂ at 37°C in DMEM containing 1% fetal bovine serum, as well as penicillin and streptomycin and 1% L-glutamine. The cell lines were derived from patients with squamous cell carcinoma of the larynx (n = 6), oral cavity (n = 3), and oropharynx (n = 1). The 10 cell lines in this study were selected from a panel of 23 cell lines that had been analyzed for in vitro sensitivity or resistance to cisplatin (8). The five most sensitive (ID₅₀ values < 5 μmol/L) and the five most resistant cell lines (ID₅₀ values > 10 μmol/L) were selected for the study.

Normal tissue samples from the nasopharynx, oropharynx, tongue, buccal mucosa, submaxillary gland, and the base of the tongue were obtained from the Department of Pathology, Spectrum Health Hospital (Grand Rapids, MI). The study was approved by the Internal Review Boards of the University of Michigan, Van Andel Research Institute, and Lund University Hospital.

**DNA and RNA Isolation.** Genomic DNA and total RNA were isolated simultaneously from the five nasopharyngeal carcinoma cell lines and from autopsied normal tissues with TRIzol Reagent (Life Technologies, Inc., Caithurgers, MD), according to the manufacturer’s instructions. Subsequently, poly(A)⁺ mRNA was isolated from the total RNA with the Oligotex mRNA mini kit (Qiagen, Inc., Valencia, CA). From the peripheral leukocytes, high molecular weight DNA was extracted with the Blood DNA Maxi kit (Qiagen, Inc.). Finally, the quality and quantities of the DNA and RNA samples were verified with agarose gel electrophoresis.

**Metaphase Comparative Genomic Hybridization.** Comparative genomic hybridization was done essentially as previously described (16) with some modifications. Briefly, before labeling, tumor DNA samples, as well as reference DNA, were digested into fragments of 100 to 2000 bp by overnight incubation with DpnII (New England Biolabs, Inc., Beverly, MA) at 37°C and checked on a 2% agarose-gel. The DNA fragments were purified with Qiagenc Quick PCR purification kit (Qiagen, Inc.), and 1 μg of digested DNA was directly labeled by the Universal Linkage System (ref. 17; Kreatech, Amsterdam, the Netherlands), according to the manufacturer’s protocol. The labeled DNA (reference DNA labeled with rhodamine and tumor DNA with dGreen) was combined and concentrated with a Microcon YM-30 column (Amicon, Bedford, MA), and 20 μL of Cot-1 DNA (Life Technologies, Inc.) were added before etomidate precipitation. The pellet was air-dried and resuspended in 10 μL of hybridization buffer (Vysis, Inc., Downers Grove, IL). Labeled DNA was denatured and applied onto denatured metaphase slides of normal lymphocytes (Vysis, Inc.). After hybridization at 37°C overnight, the slides were washed in 0.4× SSC/0.1% Tween 20 at 73°C for 4 minutes and in 4× SSC/0.1% Tween 20 at room temperature for 1 minute and then dehydrated through an etomide series. After air-drying, the slides were counterstained with 0.15 μg/mL 4′,6-diamidino-2-phenylindole in a antifade solution (Vector, Inc., Burlingame, CA). All reasonably straight, nonoverlapping chromosomes from 15 to 20 metaphases were analyzed on a Zeiss Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany) epifluorescence microscope, and images were captured and analyzed with a cooled charged-coupled device camera (Sensys Photometrics Ltd., Tucson, AZ) and software Isis version 99/4.6 (Meta Systems, Herts, United Kingdom). Chromosome regions were considered to be lost if the green/red fluorescent signal ratio was < 0.85, and signal ratio > 1.15 was considered to be gained. All gains and losses observed are summarized in Fig. 1.

**Spectral Karyotyping.** Metaphase chromosomes were obtained according to standard procedures. Spectral karyotyping was done according to the protocol included in the spectral karyotyping kit (Applied Spectral Imaging, Migdal

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**Table 1** Clinical and MTT assay data on 10 established UMSCC cell lines

<table>
<thead>
<tr>
<th>UMSCC</th>
<th>[ID₅₀ (μmol/L)]</th>
<th>Gender</th>
<th>Site</th>
<th>Tumor-node-metastasis</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14A</td>
<td>3.2</td>
<td>F</td>
<td>Oral cavity</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>17B</td>
<td>4.0</td>
<td>F</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>23</td>
<td>3.2</td>
<td>F</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>NED</td>
</tr>
<tr>
<td>46</td>
<td>3.0</td>
<td>F</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>74B</td>
<td>4.8</td>
<td>M</td>
<td>Oral cavity</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>Resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>60</td>
<td>M</td>
<td>Oropharynx</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>6n</td>
<td>36.7</td>
<td>M</td>
<td>Oral cavity</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>10B</td>
<td>23.7</td>
<td>M</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
<tr>
<td>81B</td>
<td>10.2</td>
<td>M</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>LFU</td>
</tr>
<tr>
<td>25</td>
<td>18.7</td>
<td>M</td>
<td>Larynx</td>
<td>T,N,M₀</td>
<td>DoD</td>
</tr>
</tbody>
</table>

* Data from Bradford et al. (8).

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide; DoD, dead of disease; NED, no evidence of disease; LFU, lost to follow-up.
Haemek, Israel) on freshly prepared metaphase slides to look for any subtle cytogenetic abnormality. Image acquisition was done with a SD200 Spectracube system (Applied Spectral Imaging, Migdal Haemek, Israel) mounted on a Zeiss Axioskop microscope with a custom designed optical filter (SKY-1, Chroma Technology, Brattleboro, VT). For each case, between 10 and 20 metaphases were analyzed with the spectral karyotyping view software version 2.1 (Applied Spectral Imaging).

**cDNA Microarray Expression Array Analysis.** cDNA microarrays were fabricated as described previously (18). In brief, cDNA clones of the Sequence Validated Human cDNA Library (ResGen, Invitrogen Corp., Carlsbad, CA) were amplified by PCR with primers complementary to the vector sequences. The purified PCR products were then robotically arrayed onto polylysine-coated microarray slides, on which the DNA was immobilized by UV light. The cDNA microarrays described here contained 21,632 sequence-validated human cDNA, generally with insert sizes of 0.25 to 2.5 kb.

For each experiment, mRNA of each cell line was analyzed with equally mixed mRNAs from the mucous membranes of nasopharynx, oropharynx, tongue, buccal mucosa, submaxillary gland, and the tissue of base of the tongue as a control.

![Comparative genomic hybridization data from five cisplatin resistant and five sensitive UMSCC cell lines.](image1)

Fig. 1 Comparative genomic hybridization data from five cisplatin resistant and five sensitive UMSCC cell lines. In general, more gains and losses were observed in the resistant ones, as vastly all chromosomes were involved in most of the individual cell lines. For example, losses of 3p, 8p, 10p, and short arm of the X chromosome were observed in four of five resistant cell lines and losses of 4p and 18q in all 5. Chromosomal gains were detected in 5p, 8q, 9q, 11q, and 14q in four of five resistant cell lines and gains in 3q, 7p, and 20q in all five.

![Gene expression of TIMP-2 (A) and TIMP-3 (B) in the 10 UMSCC cell lines.](image2)

Fig. 2 Gene expression of TIMP-2 (A) and TIMP-3 (B) in the 10 UMSCC cell lines. The data confirm the lower expression of TIMP-2 and TIMP-3 in the resistant cell lines.
reference. Two micrograms of poly(A)<sub>(Amicon)</sub>, the Cy3- and Cy5-labeled cDNA probes were

<table>
<thead>
<tr>
<th>UMSSC14A</th>
<th>57−69 &lt;3n&gt;, +del(X)(q10), der(1)(14;X), der(19;1); der(2)(18;2); der(2)(X;2)q21, dup(5)(q13)(q14)x2, +5 der(6)(t(6;21)), +7, +i(7)(q10), −9; der(10)(18;10), der(11)(11;13), dup(1)(q31)q5, der(13)(19;13)x2, der(14)(X;14)q21, i(18)(p10)x3, +20; der(16)(t(9;21))(p10)q10, der(21)(p9;21), +i(10)q10[p10]</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSSC17B</td>
<td>46−48; X, del(2)(q24), +der(3)(13;12), der(4)(t(7;4)), der(4)(t(4;21)), +der(5)(p;5.21), i(6;8)(q26;q24), +11, i(12)(q10)q10, der(14;14)(q10)q10, −15; der(2)(t(21;X))p8</td>
</tr>
<tr>
<td>UMSSC23</td>
<td>65−68 &lt;3n&gt;+. X, del(2)(q10)x2, der(1)(3;1)x2, +der(1)(7;1), +del(2)(q24), −3; der(3)(13;8), der(4)(t(4;5)), i(4)(p10), der(5)(5;2), der(6)(t)(6;15), der(6)(t)(9;15;6), +der(7)(7;1), +der(7)(7;X;7), del(8)(q5;8;X), i(8)(q10), i(9)(q9)(p10), der(10)(3;10), −11; der(11)(14;11), −13; der(15)(5;15), der(16)(t;6;1), +der(16)(t;6;5), +der(17)(t;17;2)x2, der(17)(t;21;17), −18; del(10)(q10), −19, −21, −22; cp11</td>
</tr>
<tr>
<td>UMSSC46</td>
<td>40−47; X, +der(1)(17), der(2)(2;5), der(4)(t(5;4)), i(5)(q10), der(5)(5;4), +der(6)(t;21;6), der(7)(7;6), der(8)(10;8), +der(9)(t;9;19), der(10)(4;10), der(11)(t;9;11), der(12)(t;12;5), der(14)(X;14)(p10), der(16)(t;18;20;cp12)</td>
</tr>
<tr>
<td>UMSSCC74</td>
<td>37−47; X, der(4)(t(4;5)), +der(5)(5;4), der(9)(t;10;8), +der(9)(t;16;9), der(14)(15)(q10)q10, der(16)(q10)q10[p10]</td>
</tr>
</tbody>
</table>

Resistant cell lines (n = 5)

| UMSSC6   | 68−71 <3n>, +der(X)(11;1:X;1)x2, der(Y)(9;Y)x2, der(1)(t;1;8;1), +del(5)(q10)x2, +7, +10, der(11)(t;11X), +12, der(14)(t;19;14)x2, −14, der(15)(t;8;15)x2, −18, −19, +20, −21; der(22)(22q10)q10x2, +22 |
| UMSSCC10B| 56−62 <3n>, X, der(X)(2;7;X); der(2)(t;2;8), der(3)(t;7;3), der(4)(t;8;4), der(5)(t;5;11), der(5)(t;5;16), del(5)(q10), ider(5)(q10)q10, der(6)(t;6;10), der(7)(t;7;9), der(7)(t;7;21), +i(7)(q10)x2, der(8)(8;11), der(8)(t;12;8), i(9)(p10), der(10)(t;10;13), der(10)(t;10;5), der(11)(t;11;5), der(11)(t;20;11;17), der(11)(t;11;8), −12, der(13)(t;13;9;13), −13; der(14)(5;14;15), der(14)(14;21)q21q10, der(15)(t;3;15), der(18)(q10), −21, −22; cp14 |
| UMSSC25  | 58−62 <3n>, +X, der(X)(X;22), der(2)(t;19;2)x4, der(3)(t;3;5), der(4)(t;10;4), der(4)(t;4;5), i(5)(q10)x2, i(5)(p10)x2, der(6)(t;22;6), der(7)(7;9)x2, der(8)(t;14;8), der(8)(t;8;14;13), −9; der(10)(q10)x10, der(10)(t;10;6), der(11)(t;16;11)x2, der(12)(9;12), −13; der(14)(5;14;15), der(14)(14;15;14), −15, −18, −19x2, der(19)(t;19;11), −21, −22x3; cp8 |
| UMSSC38  | 69−73 <3n>, +X, der(X)(X;7)x2, der(Y)(22;Y), der(1)(t;1;20;1), ider(1)(q10;10), 2, der(2)(t;4;2), der(2)(t;10;2), der(3)(t;17;3), der(3)(t;3;13), der(3)(t;3;15), der(4;9;14;9), der(4;10;4), del(5)(q10)x2, der(5)(t;5;10), der(5)(t;5;19), +der(5;9;5)x2, der(6;9;x2, der(7)(7;4), dup(7)(q22q31), der(8)(t;8;4)x2, der(8;22;8), der(9)(t;5;9)x2, der(9)(p10), der(11)(11;7;3), der(11)(11;7;3), der(11)(11;7;3), +12; der(13)(t;19;13), der(13)(t;3;13), der(13)(16;13), +der(14)(t;19;14), der(15)(t;15), der(16)(5;15), der(17)(15;9;15), −16, −17, −18; der(19)(19;4)x2, der(21)(t;18;21), −22; cp11 |
| UMSSCC81B| 48−73 <3n>, +X, der(X)(X;5;X), der(Y)(22;Y)x2, +3, −der(4;11;4), del(5)(q10), i(5)(p10), +7, +8, i(9)(p10), i(9)(q10), der(9)(t;16;9), i(10)(q10), der(11)(t;11;5), der(12)(t;12;5), der(15)(t;5;15), der(15)(t;5;15), −16, der(17)(t;17;17), del(18)(q10), +20; der(21;22)(q10)q10, cp12 |

NOTE. In general, more complex structural aberrations were seen in the resistant cell lines.

cDNA Microarray Data Analysis. Images were analyzed with the software Genepix Pro 3.0 (Axon, Union City, CA). The local background was subtracted for all spots. Any spots whose background-subtracted intensity in either the Cy5 or Cy3 channel was <150 were excluded from the analysis. The ratio of Cy5 intensity to Cy3 intensity was calculated for each spot and represented tumor RNA expression relative to the noncancerous kidney tissue pool. Ratios were log-transformed (base 2) and normalized so that the median log-transformed ratio equaled 0. Gene expression values that had expression ratios that varied at least 2-fold in all three experiments and had values of the maximum ratio minus minimum ratio > 2 were selected for the global cluster analysis. The gene expression ratios were median-centered across all samples. Gene expression values were manipulated and visualized with the CLUSTER and TREEVIEW software (M. B. Eisen<sup>10</sup>). The correlation distances were calculated as 1−r, where r indicates the Pearson correlation coefficient (20). This coefficient equals 1 for a

<sup>10</sup> Internet address: http://rana.lbl.gov.
perfect correlated series and -1 for a series that show no correlation.

The CIT software was used to find genes that were differentially expressed (with Student’s t test) between two groups of cell lines: resistant and sensitive (21). In this study, the patient groupings were based on clinical parameters. To find significant discriminating genes, 10,000 t-statistics were calculated by randomly placing samples into two groups (22). A 99.5% significance threshold ($P < 0.05$) was used to identify genes that could significantly distinguish between these two groups versus comparisons of any random groupings.

Reverse Transcriptase-PCR Analysis. To confirm the differential expressions among the paired cell line/reference mixture, reverse transcriptase-PCR was performed on two genes: tissue inhibitor of metalloproteinase 2 (TIMP-2) and TIMP-3, with Superscript one-step reverse transcriptase-PCR with platinum Taq (Life Technologies, Inc.). The primers were as follows: (a) TIMP-2, 5'-TTGATGCGAGGCGAAGAACT-3' (forward primer) and 5'-CACCACCCAGAAAGAGC (reverse primer); and (b) TIMP-3, 5'-GCATAATTACAGCATGTA (forward primer) and 5'-CCTCCCCACAGACTCTCTC (reverse primer). The expression levels of both genes were normalized against the expression of b-actin (Fig. 2A and B).

Patients. From previously analyzed clinical material consisting of 51 patients with SCCHN that received induction chemotherapy (23), paraffin-embedded specimens from 29 individuals were available for analysis of c-met expression (immunohistochemistry) and ploidy status (flow cytometry). The specimens, consisting of pretreatment biopsies and immunohistochemical examination and one for flow cytometry analysis. The tumors originated from the hypopharynx ($n = 9$), oropharynx ($n = 8$), oral cavity ($n = 7$), and larynx ($n = 5$). One of these patients had a stage 2 SCCHN, 11 patients had stage 3 tumors, and the remaining 17 had stage 4 tumors. Six of the patients were women and 23 were men. The median age of the patients was 62 years (range, 34 to 80 years). Patients were treated at Department of Oncology, University Hospital (Lund, Sweden) in 1984 to 1987 as follows: three courses of cisplatin (100 mg/m$^2$) and a subsequent 120-hour infusion of 5-fluorouracil (1000 mg/m$^2$ per 24 hours) repeated every 3 weeks, followed by radiotherapy and, if indicated, salvage surgery. Tumor response was assessed 2 to 4 weeks after completion of chemotherapy by clinical examination, computer tomography of the head and neck region, endoscopy and palpation under anesthesia, and biopsies of suspected residual tumor. Complete response was defined as disappearance of all clinically evident disease (primary tumor and node metastasis) for at least 4 weeks. Fifteen of the 29 patients (52%) died within 6 to 34 months after their diagnosis. Median follow-up was 27 months (range, 18 to 57 months).

Participation of the patients in the clinical part of the study was approved by the Ethical Committee, Lund University.

Immunohistochemical Staining of Met Oncoprotein. Sections of the primary tumors were prepared for immunohistochemical staining as described by ref. 23. Sections were stained for the expression of Met with a polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). The images of the sections were made with a Zeiss 510 confocal microscope. Multiple images of the tumor and adjacent normal re-
gions (where available) were obtained. The images were analyzed with two different quantification methods to measure the level of Met expression of the tumors. First, the images were analyzed with Image Pro Plus software (Media Cybernetics, San Diego, CA). The Met expression of the tumor regions of the image was measured on an intensity scale of 15 to 255. Intensity values below 15 were excluded because they corresponded to area lacking tissue. The final intensity of each case was determined by using a weighted pixel intensity value similar to the method described by Klineberg et al. (24). Second, the images were printed with a dye sublimation printer and were visually analyzed for Met expression on a scale of 0 to 5 (lowest to highest expression). The cases were divided into the six categories by qualitative criteria and objective discrimination by intensity profile. Cases were divided by units of ~20%. The −1 group showed no positive staining: 1 to 20% are 0; 21 to 40% are +1, 41 to 60% are +2, 61 to 80% are +3; and >81% are +4.

Flow Cytometry. Fifty-micrometer sections from tumor containing paraffin-embedded specimens were disintegrated and studied with flow cytometric DNA analysis as earlier described (25). Samples with single cell populations were classified as diploid, and those with more than one as nondiploid. In nondiploid samples, the DNA index for the abnormal cell population was calculated with the first (diploid) peak as reference.

Fig. 4 cDNA microarray data from five cisplatin resistant and five sensitive UMSCC cell lines. Supervised clustering identified some 60 genes that were expressed significantly different in the two subsets of cell lines, among which genes that are known to be involved in proliferation, metastasis, and drug resistance. The proto-oncogene c-met showed low expression in the sensitive cell lines, which in turn was the rationale to investigate its protein expression in the clinical material (arrow).
RESULTS

Cell Lines

Cisplatin Sensitivity. Five cisplatin-sensitive cell lines [UMSCC 14A, 17B, 23, 46, and 74B; ID$_{50}$ mean value 3.6 μmol/L (range, 3.0 to 4.8 μmol/L)] and five cell lines that are highly resistant to cisplatin [UMSCC 6, 10B, 25, 38, and 81B; ID$_{50}$ mean value 29.9 μmol/L (range, 10.2 to 60.0 μmol/L)] were selected for the study (Table 1).

Chromosomal Abnormalities by Metaphase Comparative Genomic Hybridization and Spectral Karyotyping. Summaries of chromosomal changes by comparative genomic hybridization and spectral karyotyping of all 10 UMSCC cell lines are shown in Fig. 1 and Table 2, respectively. In general, both sets of analyses clearly show that the chromosomal copy number and chromosomal abnormalities in the resistant cell lines far exceed those in the sensitive cell lines. In the comparative genomic hybridization analysis, almost all chromosomes revealed gains or losses in the resistant cell lines, and many loci were involved in all five cell lines (losses in 4p and 18q; gains in 3q, 7p, 11q, and 20q). In the spectral karyotyping analysis, complex structural chromosomal changes were observed, including amplifications, deletions, insertions, translocations, and so on (Fig. 3). In general, more complex aberrations were observed in the resistant cell lines.

mRNA Expression with cDNA Microarrays. Supervised clustering analysis identified ~60 genes that differed between resistant and sensitive cell lines (Fig. 4). Some of these genes are known to be associated with tumor proliferation, metastasis, and drug resistance, e.g., TIMP-2, CAVEOLIN-2, PRAME, and DAP. One of them, the MET oncogene, had relatively higher expression in the five resistant cell lines. Additional immunohistochemical results of met on primary tumors are described below.

Reverse Transcriptase-PCR. The reverse transcriptase-PCR results (Fig. 2) confirmed the microarray expression results. Fragments of 10 genes were amplified with β-actin. Both Timp-2 and Timp-3 have lower expression in the resistant cell lines than in the sensitive cell lines (Figs. 5 and 6).

Patients

Immunohistochemical Studies. The subjective and quantitative data measuring Met expression were correlated with the Spearman rank-sum test. The correlation coefficient of the two data sets was 0.9285, with an associated P value of $1.9 \times 10^{-7}$, indicating a strong correlation between the two methods. Fig. 6A depicts the expression of all of the cases in the cohort and ranks them from highest expression to lowest. Both methods indicated that cases 14, 19, and 6 expressed the highest Met intensity, whereas cases 21, 23, and 25 expressed the lowest Met intensity (Fig. 6B). Fig. 6B depicts the grouping of representative cases by weighted average intensity.

Met Expression versus Ploidy Status. Twenty of 29 tumors (67%) showed high c-met expression and the remaining 9 (31%) low expression, as measured by immunohistochemistry. By flow cytometry, 21 of 29 (72%) were nondiploid and 8 of 29 (28%) diploid (Table 3).

There was a strong relation between c-met expression and ploidy status. Six of 9 cases (67%) with low expression of c-met protein were diploid, as compared with 2 of 20 cases (10%) with high expression ($P = 0.004$, Fisher’s exact test).

Met Expression and Ploidy Status versus Response to Chemotherapy. Five of 9 (56%) patients with tumors that showed low c-met expression responded completely to induction chemotherapy. In contrast, only 4 of 20 (20%) patients with high c-met expression levels responded completely ($P = 0.088$, Fisher’s exact test; Fig. 5).
Table 3  SCCHN patients with tumors showing low c-met expression tended to have higher frequencies of complete response to induction chemotherapy than tumors with high c-met expression ($P = 0.09$, Fisher’s exact test)

<table>
<thead>
<tr>
<th>Ploidy status</th>
<th>c-Met</th>
<th>Response to CR</th>
<th>Chemotherapy PR/NR</th>
<th>Total</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid Low</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid High</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondiploid Low</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondiploid High</td>
<td>4</td>
<td>14</td>
<td>18</td>
<td>29</td>
<td>0.026</td>
</tr>
</tbody>
</table>

NOTE. In nondiploid tumors, low c-met expression correlated to complete remission ($P = 0.026$, Fisher’s exact test).

Abbreviations: CR, complete response; PR, partial response; NR, no response.

Note, although, that in a subgroup consisting of SCCHN patients with nondiploid tumors, all those with low expression of c-met ($n = 3$) responded completely to induction chemotherapy. In contrast, only 4 of 18 (22%) patients in this subgroup with tumors showing high c-met expression had such a response ($P = 0.026$, Fisher’s exact test; Table 3).

There was no association between ploidy status alone and response to chemotherapy.

DISCUSSION

In the present study, we analyzed five cisplatin-sensitive and five cisplatin-resistant cell lines by comparative genomic hybridization, spectral karyotyping, and cDNA microarray analysis, with the purpose of screening for biomarkers that may be used to predict the outcome of therapy for SCCHN. The findings

Fig. 6  Met protein expression in 29 patients with SCCHN. The cases identified in A were the three showing the highest expression for Met (14, 19, and 6) and the three with the lowest expression (21, 23, and 25). The case numbers are along the X axis and the pixel intensity levels are in the Y axis. B. The rankings for all cases in the cohort are shown from highest (brightness) weighted pixel intensity to lowest. This cohort was evaluated by semiquantitative methods and weighted pixel intensity methods and the correlation was 0.9339.
were then tested on diagnostic biopsies from 29 patients that received induction chemotherapy for SCCHN.

Complex genetic abnormalities by comparative genomic hybridization and spectral karyotyping analysis, i.e., regions of losses and gains by comparative genomic hybridization and structural changes by spectral karyotyping, were observed predominantly in the resistant cell lines. Examples of this were gain of 11q and loss of 18q, both of which have earlier been correlated to poor prognosis in SCCHN (26, 27). This could imply that drug resistance is mediated by a number of genetic changes, which would in turn indicate that altered expression profiles could be a useful tool in predicting drug resistance.

Our gene expression microarray data clearly distinguished cisplatin-resistant cell lines based on some 60 genes, many of which are known to be involved in tumor progression and metastasis (Fig. 4). These include caveolin-2, tissue inhibitor Timp-2 and Timp-3, and connective tissue growth factor, all showing higher expression in the resistant cell lines. Caveolin-2 has been reported to be up-regulated in esophageal squamous cell carcinoma (28) and Timp-2 to have a role in the initiation of breast cancer (29). Overexpression of connective tissue growth factor has been associated with attenuated cell growth in oral squamous cell carcinoma-derived cell lines (30). This is in line with the present findings because favorable response to cisplatin is preferentially seen in tumors with high proliferation rates.

A subset of genes were up-regulated in sensitive cell lines (Fig. 4), among which were preferentially expressed antigen in melanoma and calmodulin-dependent protein kinase 1. High expression of preferentially expressed antigen in melanoma has been reported in hematologic malignancies (31), and inactivation of death-associated kinase, which is a calmodulin-dependent kinase, has recently been reported to be associated with advanced stage non–small-cell lung cancer (32). The present study indicates that cisplatin-based chemotherapy could be a useful complement to traditional treatment for patients with these tumors. A biomarker of poor prognosis or advanced disease may at the same time be a predictor of favorable outcome of an alternative treatment, as is the case for her-2 in breast cancer (33, 34) and cyclin D1 in SCCHN (5, 11, 35).

Met tyrosine kinase (Met), the receptor for hepatocyte growth factor/scatter factor, is important in a variety of cell functions, e.g., proliferation, mitogenesis, angiogenesis, and metastasis. Overexpression of Met has been reported in breast, ovarian, thyroid, pancreatic, brain, and gastrointestinal tumors, and we have shown c-met overexpression to be correlated with poor prognosis in nasopharyngeal carcinoma patients (36). The latter finding is in accordance with the present, that low c-met expression is associated with cisplatin sensitivity, because most nasopharyngeal carcinoma patients receive cisplatin-based chemotherapy in combination with radiotherapy as the primary treatment. In conclusion, our data indicate that nasopharyngeal carcinoma patients with tumors having high expression of c-met may not be good candidates for concomitant chemoradiation. They might benefit from alternative primary treatment strategies.

Because low Met expression mainly was observed in chemosensitive UMSCC cell lines, by cDNA microarray, we evaluated this parameter by immunohistochemistry on paraffin-embedded tumor specimens in a well-defined clinical trial. The study population consisted of 29 HNSCC patients who received induction chemotherapy (cisplatin and 5-fluorouracil) before primary radiation therapy and who had been carefully evaluated regarding tumor response after each modality of the treatment. This cohort was a portion of a larger population of 51 SCCHN patients from which paraffin-embedded specimens were available (23). In the present study, we found that low c-met expression correlated with complete response to induction chemotherapy (cisplatin + 5-fluorouracil) in nondiploid cases (P = 0.026, Fisher’s exact test). The present findings of low expression of Met being correlated to favorable response to cisplatin regimens is of special interest in the context of a recent publication showing that adenovirus inhibition of Met expression in turn inhibits tumor growth and node metastases in prostate cancer cells (37). Therefore, targeting the Met pathway with gene therapy in combination with chemotherapy may offer a new therapeutic regimen.

Taken together, data from our comparative genomic hybridization and spectral karyotyping analysis indicate that cisplatin resistance is multifactorial and that novel drug targets must be defined to improve tumor response. Our cDNA microarray findings support the hypothesis that genetic profiling in general, and immunohistochemical detection of met expression, in particular, can be used to identify individuals with SCCHN that have a high likelihood of response to chemotherapy. These analyses, used on diagnostic biopsies, have the potential for becoming predictive tools in the management of the disease. Today, tumor-node-metastasis staging and tumor site are the basis when deciding upon treatment strategies, and no biological predictive markers have thus far been introduced into clinical trials. However, we have previously reported that cyclin D1 overexpression correlates with complete response to induction chemotherapy (5), and it might be valuable to construct a profile of several biomarkers, e.g., cyclin D1 and met, and study their impact on clinical response, as done for prognosis by cyclin D1, p16, and c-myc (35). Today, concomitant chemoradiation is gaining support in treatment of SCCHN in comparison with single-modality radiation therapy (4). However, because the combination of chemotherapy and radiation therapy is accompanied by higher morbidity, it will most likely be of great importance to individualize treatment based on analysis of biomarkers that can identify the responders before treatment starts.

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REFERENCES


Genetic and Expression Profiles of Squamous Cell Carcinoma of the Head and Neck Correlate with Cisplatin Sensitivity and Resistance in Cell Lines and Patients

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