Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]

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Chapter 8

Genetic Abnormalities Associated with Nodal Metastasis in Head and Neck Cancer

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Genetic Abnormalities Associated with Metastasis

Abstract

Background. Lymphatic metastasis represents the single most important clinical prognostic factor in head and neck squamous cell carcinoma (HNSCC), but underlying genetic mechanisms remain ill defined. Genetic differences between primary carcinomas and their corresponding metastases might form a key to understanding the metastatic phenotype. In this study we aimed to characterize such differences using a genome-wide screening measure.

Methods. Four human cell lines (MDA-686tu, MDA-686Ln, MDA-1386tu, MDA-1386Ln) derived from primary tumor and synchronous lymph node metastasis of two cases of metastatic HNSCC were subjected to comparative genomic hybridization (CGH) by differentially labeling DNA from tumor tissue and normal tissue with fluorescent agents. The labeled DNAs were simultaneously hybridized onto normal metaphase chromosomes. In addition, modified CGH was performed by directly hybridizing labeled primary tumor DNA against differentially labeled metastatic tumor DNA, allowing the direct detection of copy number differences in individual pairs. Image analysis for fluorescence intensity along the entire length of each metaphase chromosome allowed generation of a color ratio, which was used to detect copy number changes.

Results. In both cases, significant overlap was found between chromosomal aberrations present in the primary tumor and the corresponding nodal metastasis. However, several abnormalities differentiated primary tumors from their metastases. Modified CGH identified several genetic aberrations that were not detectable with the conventional CGH analysis. Gains at chromosomes 10p11-12 and 11p and deletions at chromosomes 4q22-31, 9p13-24, and 14q differentiated nodal metastases from the corresponding primary tumors in both cases.

Conclusions. The combination of conventional and modified CGH analyses facilitates the identification of DNA copy number changes that might be involved in the development of a metastatic phenotype. Future research should aim at the identification of the genes involved at the identified sites of chromosomal aberration.

The development of nodal metastasis is the most important clinical prognostic factor in head and neck squamous cell carcinoma (HNSCC). The weight of this predictor is evident from the 5-year survival statistics that demonstrate a 50% survival decrease in the event of nodal metastasis. Several lines of evidence suggest the presence of genetic differences between primary HNSCC and their corresponding nodal metastases.[1-3] Although such differences might be causally linked to the metastatic phenotype, they remain ill defined.

New genetic screening methods promise to accelerate our understanding of HNSCC.[4] One of these, comparative genomic hybridization (CGH), is based on the simultaneous, competitive hybridization of differentially fluorochrome-labeled tumor and normal reference DNA to normal metaphase spreads.[5] This approach allows a genome-wide appraisal of DNA copy number abnormalities that are commonly present in malignant disease and maps these to the corresponding chromosomal loci. Although previous CGH analyses have demonstrated that HNSCCs are characterized by a consistent pattern of DNA copy number changes,[6,7] few comparative data on primary tumors and corresponding metastases are available.[8-11] Recently, a modification on
the conventional CGH (cCGH) approach, designated modified CGH (mCGH), was reported to facilitate detection of genetic differences between breast carcinomas and their synchronous metastases.[12] In contrast to the cCGH procedure, DNA extracted from metastases is competitively hybridized with DNA from the primary tumor, thereby providing a direct comparison of the genetic content of tumor and metastasis. The combined use of cCGH and mCGH is anticipated to facilitate the detection of genetic differences between primary HNSCC and corresponding metastases. However, various levels of normal tissue "dilution" present in such samples might limit the genetic comparison of clinical specimens. Therefore, the study of cell lines established from matched pairs of primary tumor and nodal metastasis might constitute a purer initial model to detect these genetic differences.

In this study, we aimed to characterize genetic abnormalities differentiating cell lines established from primary HNSCC and corresponding lymphatic metastases using cCGH and mCGH.

**Materials and Methods**

**Cell Lines**

Four human cell lines (MDA-686tu, MDA-686Ln, MDA-1386tu, MDA-1386Ln) derived from primary tumor and synchronous lymph node metastasis of two cases of metastatic HNSCC were established by one of the authors (PGS). Case MDA-686 represented a previously untreated well-differentiated squamous cell carcinoma originating in the oropharynx from a 48-year-old man (T3N2M0). MDA-1386 represented a previously untreated squamous cell carcinoma originating in the hypopharynx of a 72-year-man (T4N3M0). The cells were grown to 80% confluence at 37°C in a 5% CO₂ atmosphere in RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin. DNA extraction was performed as described previously. All cell lines were early passages (<20).

**Comparative Genomic Hybridization**

cCGH was performed as described in detail elsewhere.[13] Equal amounts (2 μg) of

<table>
<thead>
<tr>
<th>Table 1. Genetic abnormalities differentiating primary tumor and corresponding metastasis identified by conventional and modified CGH</th>
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<tbody>
<tr>
<td><strong>cCGH-primary</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>MDA686</td>
</tr>
<tr>
<td>MDA1386</td>
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<tr>
<td>Shared</td>
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Discrepancies between conventional CGH and modified CGH are bold.
tumor DNA and normal human placenta DNA were labeled with fluorescein-12-dUTP (FITC) and Texas red-5-dUTP (Perkin Elmer, Boston, MA), respectively, coprecipitated with 15 μg of cot-1 DNA (Invitrogen, Carlsbad, CA), and suspended in a hybridization mix (50% formamide/15% dextran sulfate/2X SSC). The suspension was hybridized for 3 days at 37°C onto commercially available normal metaphase chromosome spreads (Vysis, Downing Grove, IL). On completion of hybridization, the slides were washed, and the chromosomes were counterstained with 4', 6'-diamidino-2 phenylindole (DAPI) to allow their identification. For mCGH, both primary tumor cell line DNAs were labeled with Texas red-5-dUTP, and both nodal metastasis cell line DNAs were labeled with fluorescein-12-dUTP (FITC), and each corresponding pair was simultaneously hybridized on the normal metaphase spreads.

**Image Analysis**

Ten individual metaphases were captured for each case with a cooled-charge coupled-device camera attached to a Nikon Microphot-SA microscope (Morrell Instruments, Melville, NY) and processed by the Quantitative Imaging Processing System (QUIPS, Applied Imaging, Santa Clara, CA). The chromosomes were identified by 4', 6'-diamidino-2 phenylindole (DAPI) banding analysis, segmented, the local background subtracted, and the median axis 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 on the basis of 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 defined as ratios of 0.8 or less.

**Results**

**cCGH Analysis**

Overall, primary tumors and their corresponding lymphatic metastases displayed a high degree of overlap in the number and types (gain, loss, amplification) of genetic abnormalities, as depicted in Figure 1. In MDA-686 16 abnormalities and in MDA-1386 31 abnormalities were identical in the primary tumor and the corresponding metastasis.
Figure 1. Ideogram of chromosomal abnormalities identified in cell lines MDA-686 (A) and MDA-1386 (B). Bars on the left of the chromosomes represent losses, bars on the right of the chromosomes represent gains as identified by conventional CGH of primary tumor (-), conventional CGH of corresponding nodal metastasis (.....) and modified CGH (--). High-level amplifications are indicated by thickening of the bars at the corresponding loci.
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Gains were most commonly found at chromosomes 3q, 5p, 7p, 11q, and 16q. Losses most often involved chromosomes 3p, 5q, 7q, 8p, 9p, 13q, and 18q. High-level amplifications were detected at several loci (Figure 1), of which 5q and 11q13 were present in both cases. Several abnormalities were detected that differentiated primary tumors from their corresponding metastases, including 12 abnormalities in MDA-686 and 13 abnormalities in MDA-1386 (Table 1). Of abnormalities detected in primary tumors that were no longer present in the metastasis, gain of chromosome 17p was identified in both cases. Of abnormalities detected in the nodal metastases that were not present in the corresponding primary tumor, gain of chromosome 9q11-32 and loss of 4q22-31 were present in both cell lines.

**Modified CGH Analysis**

Figure 2 shows a representative metaphase spread after hybridization of tumor DNA vs metastatic DNA from case 1386. A compilation of mCGH data is shown in Figure 1. mCGH confirmed most of the abnormalities detected by cCGH. However, the comparison of this technique with cCGH showed several discrepancies as detailed in Table 1. mCGH revealed quantitative differences in copy number between abnormalities present in both primary tumor and corresponding metastasis that were not discernible with cCGH; for example, both cases harbored copy number loss at chromosome 9p in nodal metastases relative to corresponding primaries. mCGH reclassified two abnormalities in MDA-686 and four abnormalities in MDA-1386 that were initially indicated by cCGH.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Candidate genes</th>
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<tbody>
<tr>
<td>-4q22-31</td>
<td>MAPK10; mitogen-activated protein kinase 10</td>
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<tr>
<td></td>
<td>MAPK2K1IP1; mitogen-activated protein kinase kinase 1 interacting protein 1</td>
</tr>
<tr>
<td></td>
<td>NFkB1; nuclear factor of kappa light</td>
</tr>
<tr>
<td></td>
<td>polypeptide gene enhancer in B-cells 1 (p105)</td>
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<tr>
<td></td>
<td>APG-1; Heat shock protein 110 family</td>
</tr>
<tr>
<td></td>
<td>ANXA5; annexin A5</td>
</tr>
<tr>
<td>-9p13-24</td>
<td>TEK; TEK tyrosine kinase</td>
</tr>
<tr>
<td></td>
<td>p16/CDKN2A; cyclin-dependent kinase 2A</td>
</tr>
<tr>
<td></td>
<td>p15/CDKN2B; cyclin-dependent kinase 2B</td>
</tr>
<tr>
<td></td>
<td>p18/CDKN2C; cyclin-dependent kinase 2C</td>
</tr>
<tr>
<td></td>
<td>p19/CDKN2D; cyclin-dependent kinase 2D</td>
</tr>
<tr>
<td>+10p11-12</td>
<td>MAP3K8; mitogen-activated protein kinase 8</td>
</tr>
<tr>
<td></td>
<td>ITGB1; integrin, beta 1</td>
</tr>
<tr>
<td>+11p</td>
<td>HSSOX6; SOX-6</td>
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<tr>
<td></td>
<td>TSG10; tumor susceptibility gene 10</td>
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<tr>
<td></td>
<td>PAX6; paired box gene 6</td>
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<tr>
<td></td>
<td>LIM1; LIM homeobox protein 1</td>
</tr>
<tr>
<td></td>
<td>TCL2; T-cell leukemia/lymphoma-2</td>
</tr>
<tr>
<td>-14q</td>
<td>FOXG1A; fork-head box G1A</td>
</tr>
<tr>
<td></td>
<td>LGALS3; lectin, galactoside-binding, soluble, 3 (galectin-3)</td>
</tr>
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</table>

Table 2. Candidate genes mapped to recurrent loci differentiating primary tumor and nodal metastasis identified by modified CGH in HNSCC.
as differentiating primary tumor from metastasis as nonexistent, including the gains of 17p and 9q11-32 that were identified in both cases by cCGH. Furthermore, several additional genetic abnormalities differentiating primary tumors from their corresponding metastases were detected at chromosomal loci that did not show any apparent aberrations by cCGH. Gains at chromosomes 10p11-22 and 11p and losses at chromosomes 4q22-31, 9p13-24, and 14q were detected in both nodal metastases compared with the corresponding primary tumors detected by mCGH. Of these, only loss of 4q22-31 had been detected by cCGH.

**Discussion**

To identify genetic imbalances differentiating primary HNSCC tumors and their synchronous metastases, we studied four HNSCC cell lines using cCGH and mCGH.

Overall, a similar pattern of chromosomal abnormalities was seen in the cell lines relative to reports on primary HNSCC.[6,7] A high degree of concordance between genetic abnormalities in cell lines derived from primary tumors and their corresponding nodal metastases was observed, reflecting a common clonal origin. However, several abnormalities differentiated primary tumor cell lines from the corresponding metastases. These abnormalities might be involved in the onset and/or progression of a metastatic phenotype. Alternately, these abnormalities might underlie behavior differences observed between primary tumors and nodal metastases, such as a divergent response to chemoradiation treatment.

In both cases, nodal metastases displayed DNA copy number gains at 10p11-12 and 11p and DNA copy number losses at 4q22-31, 9p13-24, and 14q relative to the corresponding primary tumors. Several studies have shown these loci to be frequently altered in HNSCC.[6,14,15] In addition, loss of heterozygosity studies have further refined the minimal common genetic regions of involvement and linked several of these abnormalities to an unfavorable prognosis.[16-19] However, thus far, none of these aberrations have been previously linked to the development of nodal metastasis.

cCGH studies comparing the genetic content of clinical primary tumor and nodal metastasis specimens have been reported.[8,9,11] Although thus far ~63 cases of HNSCC have been evaluated, the comparison of previous studies yields conflicting data. This discrepancy might reflect the heterogeneous phenotype of metastatic HNSCC. On the other hand, the genetic comparison of tumor and metastasis specimens through CGH might be influenced by various levels of normal tissue "contamination" present in clinical samples. Therefore, the genetic comparison of cell lines established from primary HNSCC and synchronous metastases might constitute a purer initial model to study HNSCC metastasis. In a cell line model, the addition of mCGH to cCGH is especially powerful, because mCGH-detected DNA copy number changes between primary and metastasis are genuine and do not represent various levels of normal tissue "dilution." In our study, four of the five abnormalities associated with metastasis that were present in both cell lines were identified only after mCGH analysis. Our data also demonstrate clearly that although mCGH directly detects DNA copy number differ-
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ences between primary tumors and corresponding metastases, cCGH analysis is required to reveal the nature of these differences. An evident example of this requirement is the differential origin of 14q loss in our cases.

The chromosomal loci identified in this study harbor several interesting candidate genes that might be targets of these chromosomal imbalances (Table 2). These include mediators of the mitogen-activated protein kinase (MAPK) signal pathway and transcription factors such as NFκB1 involved in the phosphatidylinositol 3-kinase (PI3K) pathway, all of which are known to be involved in human cancer.[20,21] Other candidate genes include mediators in cell adhesion. As an example, decreased expression of galectin-3 has been reported in several human cancers, including HNSCC, and a significant role in the development of metastasis has been demonstrated.[22,23]

Although the preliminary data presented in this study are encouraging, clearly a larger number of cases is required to make definitive statements. In addition, it should be taken into account that the use of cell lines might introduce the possibility of genetic evolution in culture. However, recent molecular-cytogenetic studies addressing this issue do not show significant differences, suggesting that analysis of cell lines might be a global representation of chromosomal aberrations in primary tumors.[24,25]

Conclusion

The combination of conventional and modified CGH facilitates the identification of DNA copy number changes differentiating cell lines derived from primary HNSCC and synchronous nodal metastasis that might be involved in the development of a metastatic phenotype. Future research is needed to validate our findings and identify genes involved in the identified sites of chromosomal aberration differentiating primary and metastatic tumor cell lines.

References

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