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Increasing Levels of MYC and MET Co-Amplification During Tumor Progression of a Case of Gastric Cancer

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ABSTRACT: The cytogenetic study of a nodal metastasis from a gastric carcinoma, after two passages in nude mice, revealed a large number of double minutes. Comparative genomic in situ hybridization (CGH) analysis using DNA extracted from this xenograft revealed the existence of three clear amplification units that originated from the chromosomal subregions 6q24-25, 7q31-32, and 8q24 in the xenograft DNA. Similar, though less prominent, CGH results were found with DNAs extracted from the primary tumor and its metastasis, implying that the same amplicons were also present, albeit less abundantly, in the DNAs of these neoplastic tissues. Southern analysis of the second-passage xenograft detected 18- and 10-fold amplification of MET (located at 7q31) and MYC (located at 8q24), respectively. The retrospective study of the first passage of the xenograft, as well as of the metastatic and primary tumors before xenografting, showed amplification levels of MET of, respectively, 12-, 9-, and 5-fold and MYC of, respectively, 8-, 7-, and 5-fold. Our results suggest that increased levels of co-amplification of MYC and MET correlate with enhanced growth potential in this case of gastric carcinoma.

INTRODUCTION

The MET gene located at 7q31 is one of the protooncogenes most frequently altered in stomach cancer [1]. This protooncogene encodes a growth factor receptor controlling cell proliferation of epithelial cells of the liver and of the gastrointestinal tract [2]. Amplification and abnormal expression of the MET gene are common events in all histologic types of primary gastric cancer, as well as in gastric cancer-derived cell lines [3, 4]. A good correlation has been observed between amplification of the MET gene and advanced clinical stages and poor prognosis of patients with gastric cancer [4].

Amplification of MYC is a frequently reported phenomenon in human malignancies [5], supposedly conferring in vitro proliferative advantage to tumor cells [6, 7]. Several reports described amplification of MYC in gastric carcinomas transplanted into nude mice. However, most of these studies did not determine whether amplification of this oncogene takes place before transplantation or during passage in the nude mice [8–11].

In an attempt to determine the timing of amplification of MET and MYC in gastric cancer, we studied retrospectively a case of gastric carcinoma in which amplification of these oncogenes had been found in xenografts.

MATERIALS AND METHODS

A primary gastric carcinoma and a regional lymph node metastasis from a 39-year-old male were studied. Hematoxylin-and-eosin-stained sections were used to classify the tumor according to Laurén [12]. The growth pattern was classified according to Ming [13]. Fresh samples from the nodal metastasis were heterotransplanted into nude mice.

Fresh material from the xenograft (passage 2) was studied by conventional cytogenetics and fluorescence in situ hybridization analysis. DNA extracted from frozen material from the primary tumor, lymph node metastasis, and xenografts (passage 1 and 2) were available for Southern and comparative genomic in situ hybridization (CGH) analyses.

Tumor Heterotransplantation

Mice of the N:NIH(s)Il-nu/nu strain [14] were raised from stocks obtained from the Animal House, Medical School, University of Cape Town, Cape Town, South Africa, and were maintained under sterile conditions throughout the experiments.
Tumor specimens from the nodal metastasis were cut in pieces 4 to 5 mm in diameter and transplanted subcutaneously (s.c.) in the interscapular regions of male and female nude mice. The growth of the tumors was monitored once a week by ruler measurement of tumor length (a) and width (b). Tumor volumes were estimated by the formula: \( V(\text{mm}^3) = axb^2 \times \frac{1}{2} \).

Tumor volumes were plotted versus days after implantation and tumor doubling times (Td) calculated.

**Cytogenetic Analysis**

For chromosome analysis, the xenografted tumor (passage 2) was surgically resected and processed directly as previously described [15]. Part of the chromosome preparations was GTG-banded and part stained only with Giemsa.

**Comparative Genomic In Situ Hybridization (CGH)**

DNAs of the primary tumor, lymph node metastasis, and xenograft were isolated according to standard protocols and used for CGH analysis [16, 17] as described elsewhere [18, 19].

In short, tumor and reference (46,XY) DNAs were differentially labeled with biotin-14-dATP (BRL, Gaithersburg, MD) and digoxigenin-11-dUTP (Boehringer Mannheim, Germany), respectively, using commercially available nick translation systems. An amount of 150–400 ng of both DNAs was coprecipitated in the presence of 50–100 × Cot-1-DNA (BRL), dissolved in 10 μL hybridization mixture [20], denatured, preannealed at 37°C for 30 minutes and, finally, used for hybridization to denatured BrdU-incorporated high-resolution metaphase spreads obtained from a normal male. Hybridization was carried out at 37°C for 3–4 days in a moist chamber. Visualization of hybridizing DNAs was accomplished using Texas-Red-conjugated avidin and biotinylated anti-avidin antibodies (both from Vector Laboratories, Burlingame, CA) for biotin-labeled probes and fluorescein-conjugated sheep anti-digoxigenin (Boehringer Mannheim, Germany) and fluorescein-conjugated donkey anti-sheep (Jackson Immunoresearch, West Grove, PA) for digoxigenin-labeled probes. Slides were immersed in antifade-solution [20], supplemented with the blue DNA-specific dye DAPI (Sigma, St. Louis, MO) for (G-banded-like) counterstaining of the chromosomes.

Evaluation and accurate screening of the CGH results were attained using a Zeiss Axiophot epifluorescence microscope equipped with a Photometrics high-performance CH_370/H cooled CCD-camera (Photometrics, Tucson, AZ) interfaced onto a Macintosh Quadra 950 computer.

Image acquisition, processing, superimposition, and display (in red-green-blue pseudocolors) were accomplished using the ONCOR Image F.I.S.H. software package (ONCOR Imaging, Gaithersburg, MD). CGH analysis of recorded metaphase spreads, allowing the detection and chromosomal (sub)localization of genomic regions that are over- or under-represented in the tumor DNA as revealed via an increased or decreased Texas Red-versus-FITC (i.e., tumor-versus-normal) fluorescence ratio, was performed using the Comparative Genomic Hybridization applications within the ONCOR Image F.I.S.H. software package, as described previously [18, 19]. Photographic recording of CGH results was carried out with a Tektronix Phaser II DSX color printer.

CGH standardization experiments, using normal human DNA as test and reference DNA, revealed that for every chromosome the test vs. reference fluorescence profile virtually never deviated below 0.85 or above 1.15 (heterochromatic regions, like centromeres and telomeres, were excluded for CGH analysis [16]). For this reason we used these values as thresholds to determine the presence of DNA under- and over-representation (CGH ratio lower than 0.85 and higher than 1.15, respectively) in the tumor genome(s).

**Amplification Analysis**

High-molecular-weight DNA was isolated using standard procedures [21]. For amplification studies DNA was digested with the restriction enzyme EcoRI, electrophoresed on agarose gels, blotted onto nylon membranes, and hybridized with probe MYC exon 1 cDNA and MET probe (American Type Culture Collection, Rockville, MD). The degree of amplification was calculated by rehybridizing the blots with a control probe. COL1A2 (collagen 1 pro-2-chain). The autoradiograms were scanned using an automated scanning densitometer (LKB Gelscan XL).

**RESULTS**

**Tumorigenicity and Growth Characteristics**

The tumorigenicity rate of the neoplastic tissue retrieved from the metastasis was 100% (6/6). Subsequent to repeat transfer, a permanent tumor line was established. Total tumor transplantability for seven generations was 100%.

The time that elapsed between inoculation and the interpolated theoretical volume of 100 mm³ (the delay time to 100 mm³) varied from 46 days on passage 1 to 22 days on passage 2, for an implanted tumor fragment of 4 mm³.
Figure 2  A) Representative results from CGH with differentially labeled tumor (red) and reference normal DNA (yellow) hybridized onto a normal human metaphase spread (46,XY). Strong red signals are visible for chromosomes 6, 7, and 8. B) Measured intensity profiles for chromosomes 7 (top) and 8 (bottom). Tumor and reference DNAs are represented by, respectively, red and green lines in the graphic. The blue lines represent the CGH ratio profile.
Cytogenetics

We analyzed 10 metaphases from the xenografted tumor (passage 2). Figure 1 shows one of the metaphases showing multiple numerical and structural abnormalities, as well as multiple double minutes.

Comparative Genomic In Situ Hybridization

CGH analyses were carried out to identify and define the origin and localization of the supernumerary DNA sequences within the DNA of the tumor xenograft. In all 10 metaphase spreads examined, the hybridization patterns of tumor and normal DNAs depicted two easily discernible, overrepresented genomic regions on chromosomes 7 (7q31-32) and 8 (8q24), and a considerably less discernible one on chromosome 6 (6q24-25) (Fig. 2).

Similar results were found using DNAs of the primary tumor and its metastasis as test DNA (not shown). The level of overrepresentation of the three amplicons in DNAs of the primary tumor and nodal metastasis appeared nevertheless lower than that observed in the xenograft DNA: lower tumor-versus-normal ratios for each of the three respective chromosomal regions, and less easily and less frequently observed amplicons by CGH in the tumor and the metastasis than in the xenograft (not shown). These results imply that the level of amplification of the 6q-, 7q-, and 8q-derived genomic regions, in the DNAs of the primary tumor and its nodal metastasis, is lower than that encountered in the DNA of the xenograft.

Amplification Analysis

All tumor samples (primary tumor, lymph node metastasis, and the tumor material recovered from each of the two passages of the heterotransplanted metastasis) showed amplification of MET and MYC (Fig. 3). Amplification levels of MET were 5-, 9-, 12-, and 18-fold in, respectively, the primary tumor, metastasis (before xenografting), passage 1 of the xenograft, and passage 2 of the xenograft. Amplification levels of MYC were of 5-, 7-, 8-, and 10-fold in, respectively, the primary tumor and its nodal metastasis, lower than that observed in the xenograft DNA: lower tumor-versus-normal ratios for each of the three respective chromosomal regions, and less easily and less frequently observed amplicons by CGH in the tumor and the metastasis than in the xenograft (not shown). These results imply that the level of amplification of the 6q-, 7q-, and 8q-derived genomic regions, in the DNAs of the primary tumor and its nodal metastasis, is lower than that encountered in the DNA of the xenograft.

DISCUSSION

Gastric carcinomas are among the human epithelial tumors less frequently studied (cyto)genetically, partly at least because it is difficult to grow gastric cancer cells in vitro [11, 22]. In an attempt to overcome this limitation we have used nude mice as recipients for xenografts of gastric tumors. Recently, a similar approach was successfully used by others [8-11], enabling the establishment of gastric cancer-derived cell lines after a number of passages in nude mice. These results suggest that during heterotransplantation, (sub)populations of tumor cells acquire an intrinsic growth advantage or benefit from the absence of inhibitory signals from stromal cells present in the primary tumor.

Cytogenetically, the xenograft studied by us (passage 2) showed massive chromosome abnormalities and double minutes, a cytogenetic evidence of gene amplification [5, 23, 24].

In an attempt to localize the regions where these genetic imbalances had occurred, CGH analysis was carried out [16, 17]. This technique provides a straightforward approach for detection of gains and losses of whole chromosomes or chromosomal segments [18, 19, 25], even in the absence of conventional cytogenetic studies [17, 26].

The CGH finding of two clearly discernible amplicons originating from 7q31-32 and 8q24 in the DNA of the xenografted tumor was in keeping with the possibility that proto-oncogenes MET and MYC, which are located in 7q31 [27] and 8q24 [28-30], respectively, were amplified. This suggestion was confirmed by Southern analysis, which revealed levels of 18-fold and 10-fold amplification of MET and MYC, respectively, in the same material. The discrepancy in the levels of amplification for the two genomic regions as estimated by CGH and molecular amplification analyses is probably due to a general technical limitation of the CGH technique [16], which consists in the fact that the height of the estimated tumor-versus-normal CGH ratio does not quantitatively reflect the level of amplification in cases of increased gene copy numbers (i.e., amplicons).

Amplification and abnormal expression of MET gene has been reported in a number of gastric cancer-derived cell lines, as well as in primary gastric carcinomas [4, 31]. Preferential retention of a duplicated marker chromosome containing the amplified MET gene has been observed after endoreduplication of MNK45, a poorly differentiated gastric carcinoma cell line, suggesting a gene dosage effect along the several passages during in vitro cultures [31].

The co-amplification of the MYC oncogene observed by us in the xenograft is in keeping with previous observations of frequent amplifications of this gene in gastric carcinomas transplanted in mice [8-11].

Retrospective amplification analysis of MET and MYC showed that all specimens (primary tumor, nodal metasta-
sis, and passage 1 of the xenograft) had amplification of both genes, thus demonstrating that this phenomenon was already present in the neoplastic tissues of the patient. The increased levels of amplification of both genes in the xenografts as compared to the primary and metastatic tumor may be caused, partly at least, by a greater mixture of the tumor cells and stromal cells in the fresh samples. Alternatively, our observations may indicate the progressive selection of cells with high levels of MET and MYC co-amplification. In our opinion, the multistep increase of the amplification levels favors the latter possibility.

The higher number of copies of MET and MYC in the metastasis and passage 2 of the xenograft as compared to, respectively, the primary tumor and passage 1 in the nude mice, suggests a relationship between MET and MYC amplification and growth rate of the neoplastic cells. This hypothesis is further supported by the progressive increase of the percentage of S-phase cells, as assessed by DNA flow cytometry (data not shown), and by the observation that the delay time to 100 mm² dropped from 46 days in passage 1 to 22 days in passage 2.

Altogether, our main conclusions are: 1) In the case herein described, MET and MYC amplification was prior to heterotransplantation; 2) the levels of amplification increased from primary tumor to nodal metastasis, and along several passages in nude mice; 3) the levels of amplification of MET and MYC appear to be related with the rate of proliferation of the xenografted tumor cells in the nude mice.

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