Unknown Primary Tumors: Establishment of Cell Lines, Identification of Chromosomal Abnormalities, and Implications for a Second Type of Tumor Progression¹

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ABSTRACT

We describe the establishment of two human unknown primary tumor (UPT) cell lines and a comparison of their cytogenetic properties with two direct harvests from two tumor biopsy specimens. Histopathological analysis of all four tumor specimens revealed them to be undifferentiated adenocarcinomas of unknown origin. The chromosome numbers in these samples vary between 38 and 144. Consistent structural anomalies involving chromosome 1 were observed in all cases. These included a deletion of the short arm (4 of 4), duplication of 1q (3 of 4) and the presence of homogeneously staining region (2 of 4). Several additional chromosomal changes involving chromosomes 7, 8, and 9 were also observed, but these were less consistent and their importance is not yet clear. Abnormalities in chromosome 1 have generally been associated with advanced malignancy. The finding of consistent chromosome 1 changes in UPT supports our hypothesis that these are type 2 progressors in that benign or less malignant stages are not readily identified. In short, UPT are likely malignant soon after transformation occurs, and this is reflected in their rapid acquisition of the metastatic phenotype and abnormalities in chromosome 1. We are therefore characterizing a number of additional UPT cases to determine if chromosome 1 and other cytogenetic changes are consistently associated with this unique subgroup of tumors.

INTRODUCTION

Tumors that present as metastases without an identifiable primary neoplasm, i.e., UPT,⁴ comprise approximately 5% of malignant solid tumors (1, 2). Patients with UPT represent a disturbing clinical problem because of their poor prognosis (mean survival is 24 weeks) and their tumors' resistance to established treatment regimens. UPT have usually been considered to represent a mixture of different kinds of tumors and have not generally met treatment criteria established for tumors of known origin. It is our contention, however, that while UPT have some common cytogenetic features consistent with an advanced state of malignancy and, in addition, we describe the establishment and characterization of two UPT cell lines that have not, to our knowledge, been described previously. The implications of these findings in relation to defining tumor progression are that progression must also occur by means other than the classic description(s) of Foulds (3, 4).

MATERIALS AND METHODS

Tumor Samples. Histopathological analysis of the four tumor specimens revealed them to be undifferentiated adenocarcinomas of unknown origin. The four samples were isolated from metastases to different anatomical sites. UP-1 was isolated from a metastasis in the right antecubital fossa; UP-2 was isolated from a metastasis to a left axillary node; UP-3 was isolated from a metastasis that presented as a skin nodule; and UP-4 was isolated from a pleural effusion. None of the patients had received prior chemotherapy or radiotherapy at the time of biopsy.

Monolayer Cell Cultures. Tumor specimens UP-1 and UP-2 were obtained immediately following surgical biopsy and processed as follows. The tumors were minced into 1-mm cube fragments and dissociated into single cells by overnight treatment with 5 ml of unsupplemented RPMI 1640 medium containing 1 mg/ml collagenase type I (Sigma Chemical Co., St. Louis, MO) at 37°C. The cells were then centrifuged at 1200 rpm for 5 min and resuspended in T-25 tissue culture flasks containing 5 ml of RPMI 1640 supplemented with 5% fetal bovine serum, penicillin, streptomycin, and epidermal growth factor (5 μg/500 ml), insulin, transferrin, and selenium. The medium was changed every 4 days until the cells reached confluency, whereupon they were subcultured at 1:2 dilution. Cells were maintained in the same medium and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Mycoplasma Screening. Cells were routinely passaged a minimum of three times in antibiotic-free medium and then tested for Mycoplasma contamination with a kit supplied by Gen-Probe (San Diego, CA). Mycoplasma contamination was never observed in any of the cell lines described.

In Vitro Doubling Time. Triplicate 60-ml tissue culture dishes were seeded with 2 × 10⁶ cells in 5 ml of medium/dish. Cell counts were performed every 24 h for 7 days. Population doubling times were calculated from the resulting growth kinetics using linear regression analysis.

Tumorigenicity in Nude Mice. The tumorigenicity of cultured cells was assessed by injecting a cell suspension containing a minimum of 1 × 10⁶ cells in a volume of 0.1 ml phosphate buffered saline. The sites chosen were i.s., s.c., i.v., and i.p. UP-2 was also injected into the subrenal capsule and mammary fat pad. Tumors were removed when they reached 1 cm in diameter, whereupon they were reestablished in culture and karyotyped.

Chromosome Analysis. The cell lines UP-1 and UP-2 were harvested for cytogenetic analysis using the procedure described by Trent and Thompson (5). Briefly, monolayer cultures were incubated for 2 h at 37°C in the presence of 0.05 μg/ml demecolce (Colcemid; Sigma). The cells were then trypsinized, washed, and resuspended in 0.075 M KCl at 37°C for 20 min. UP-3 was processed as described above for UP-1 and UP-2 monolayer cell cultures. Briefly, following enzymatic disaggregation, the cells were resuspended in T-25 culture flasks in fresh medium for 48 h and then harvested for cytogenetic analysis as described above. The time of exposure to Colcemid was increased to 4

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4 The abbreviations used are: UPT, unknown primary tumor; i.s., intrasplenic; HSR, homogeneously staining region.

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Regardless of the route of injection, changes in morphology or growth rates. Tumorigenicity. The injection of 1 x 10^6 UP-1 cells into nude cell line is >40 for both UP-1 and UP-2 without any observed contrast, the cell doubling times for UP-2 remained identical with the International System for Human Cytogenetic Nomenclature (7). Detection of Intermediate Filaments. Indirect immunofluorescence for cytokeratin, vimentin, and desmin was performed using a modification of the procedure described in Osborne and Weber (8). Briefly, cells were cultured on glass coverslips, rinsed with phosphate buffered saline, and fixed with paraformaldehyde for 10 min at room temperature. Fixed cells were first incubated with the appropriate mouse antibody to human intermediate filaments, followed by fluorescein isothiocyanate-conjugated antimouse antibody. Samples were mounted with Fluoromount G (Southern Biotech, Birmingham, AL) and examined with a Zeiss fluorescence microscope.

RESULTS

In Vitro Growth Characteristics. Each cell line displayed a unique growth pattern in tissue culture. UP-1 and UP-2 both grew as monolayer cultures and exhibited cuboidal morphology typical of epithelial cells. UP-1 grew as a confluent monolayer, whereas UP-2 characteristically formed tightly compacted clusters of cells. Early passages of both cell lines exhibited mixed morphologies containing both epithelial and stromal cell types. Later passages of both cell lines exhibited only epithelial-like cells.

Growth kinetics revealed distinct doubling times for each of the cell lines (Table 1). The doubling times for UP-1 decreased from early to later passages. Early passage (p10) doubling time for UP-1 was 2.7 days, whereas later passage (p26) cultures had a doubling time that decreased significantly to 1.2 days. In contrast, the cell doubling times for UP-2 remained identical (4.2 days) from early (p9) to later passage (p26).

Plating efficiencies for each cell line were determined at passage 26 and were also distinct for each culture. UP-1 had a plating efficiency of 30%, whereas UP-2 displayed a much lower plating efficiency of 5%. Currently, the highest passage of each cell line is >40 for both UP-1 and UP-2 without any observed changes in morphology or growth rates.

Tumorigenicity. The injection of 1 x 10^4 UP-1 cells into nude mice produced tumors at all injection sites, including the spleen, subcutis, lung, and peritoneum. Cells injected s.c. formed palpable tumors within 14 days and grew at a relatively uniform rate, reaching 1 cm in size ≈30 days after inoculation. All tumors were removed, reestablished in culture, and subsequently karyotyped to confirm their human origin. Reinoculation of new hosts with cells isolated from excised tumors formed tumors at all sites. UP-1 never produced detectable metastases regardless of the route of injection.

In contrast, UP-2 cells failed to form tumors irrespective of the route of inoculation (s.c., i.v., i.s., i.p., subrenal capsule, and mammary fat pad) and in spite of observation periods exceeding 12 months. UP-2 cells were also injected s.c. into NIH-3 LAK cell deficient nude mice and again failed to form tumors.

DISCUSSION

These studies suggest that UPT are a unique group of tumors in that they manifest cytogenetic abnormalities generally associated with advanced malignancy immediately upon diagnosis. All four tumors had changes in chromosome 1 in a spectrum covering all of the described abnormalities in chromosome 1 reported by Atkin (9).

Our success rate for establishing cell lines from human UPT is ≈13% (2 of 15). We also have two additional cultures currently at less than 10 passages. Tumor formation in athymic nude mice was only observed with UP-1, and these tumors never metastasized, even after injection at multiple sites and after prolonged observation for periods exceeding 6 months. A much greater success rate has been reported for direct inoculation of malignant solid tumor specimens into athymic nude mice (10). However, direct injection of 15 UPT biopsy specimens failed to form tumors in athymic nude mice irrespective of the route of inoculation (s.c., i.v., i.s., i.p., and subrenal capsule) and at inoculum doses exceeding 1 x 10^7 cells (data not shown).

Each of the cell lines established from UPT displayed significant phenotypic diversity. Although both cell lines were from histologically similar tumors, we expected UPT to display phenotypic diversity based on their probable heterogeneous organ origin. This heterogeneity was, in fact, observed as differences in growth (and kinetic) properties, tumorigenicity, morphology, and karyotype. Of interest was the fact that within each cell line and its clones, we did not observe any significant heterogeneity with respect to karyotypic changes, growth, and kinetic properties or tumorigenicity (data not shown).

Karyotype Analysis. The four tumors displayed highly aneuploid karyotypes. Three of them (UP-1, UP-3, and UP-4) displayed a modal chromosome number near 60, whereas UP-2 was hypodiploid, with a modal chromosome number of 44 (Table 2). The structural chromosome alterations of the two established cell lines and two direct preparations from fresh biopsies are presented in Table 2 and Figs. 1-4. Structural alterations involving chromosome 1 were observed in all four cases. Clonal alterations in chromosome 1 included nonreciprocal translocations resulting in the loss of the short arm, iso- chromosome for the long arm, and evidence for gene amplification in the form of HSRs. An additional clonal structural alteration seen in two of four of the tumors was a simple deletion of the long arm of chromosome 7 (UP-1 and UP-2). Other chromosomes, including 8 and 9, were also invariably rearranged. In addition to these identifiable marker chromosomes, unidentified marker chromosomes were present in all four tumors.

Karyotypic information from fresh tumor specimens from which the cell lines were established was not obtained. However, a comparison of early (p3) to later passages (>20) demonstrated that these cells were stable and did not display significant karyotypic “evolution” (as determined by the retention of marker chromosomes without generation of new karyotypic alterations). Significantly higher numbers of chromosome alterations were not observed in the two cell lines as compared...
CHROMOSOMAL ABNORMALITIES IN UNKNOWN PRIMARY TUMORS

Table 2 Summary of cytogenetic analysis of UP-1, -2, -3, and -4

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of cells counted</th>
<th>Range Mode</th>
<th>Identification of clonal structural alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP-1</td>
<td>50</td>
<td>40-112</td>
<td>58 M1 = t(1;14)(p13q12) M2 = del(7)(q11) M3 = del(11)(q23-p13) M4 = i(11q) M5 = t(17;1)(p13q21) M6 = del(3)(q13)</td>
</tr>
<tr>
<td>UP-2</td>
<td>50</td>
<td>38-97</td>
<td>58 M1 = t(1;14)(p13q11) M2 = qHSR(q42-q21::HSR::cen) M3 = del(7)(q11) M4 = i(8q) M5 = t(9;9)(p11q11) M6 = t(13;7)(q34;7)</td>
</tr>
<tr>
<td>UP-3</td>
<td>50</td>
<td>45-144</td>
<td>65 M1 = i(q1) M2 = t(2;7)(q37;?) M3 = ins(3)(q29-q23::7q23-p27) M4 = t(3;2)(q25q14) M5 = ins(6)(q27q12;?::812-p25) M6 = t(12;7)(q24;7) M7 = dup(12)(q24-q22) M8 = t(13;10)(p13q11) M9 = U-mar</td>
</tr>
<tr>
<td>UP-4</td>
<td>50</td>
<td>42-114</td>
<td>57 M1 = pHSR(q44-p21::HSR) M2 = t(2;7)(q12) M3 = t(4;7)(35;?) M4 = 6qHSR(p25-q27::HSR::?) M5 = t(10;7)(p15;7)</td>
</tr>
</tbody>
</table>

* U-mar, unidentifiable marker chromosome.

to the direct harvest preparations from the two fresh tumor specimens.

Intermediate Filament Expression. The cell lines expressed cytokeratins, at both early and later passages, as determined by indirect immunofluorescence using a monoclonal antibody specific for keratin (see Table 1). Detection of vimentin was also observed at both early and late passages; however, as expected, neither cell line expressed desmin (Table 1).

Detailed chromosome banding analysis of each of the two cell lines and two additional direct harvests from tumor biopsy specimens revealed interesting structural and numerical karyotypic alterations (see Table 2, Figs. 1-4). However, a comparison of the cytogenetic properties of UPT demonstrated a number of similar karyotypic alterations that warrant further discussion.

Our goals for characterizing UPT by detailed banding analysis were 3-fold: (a) to determine if UPT retained tumor specific alterations that would allow us to identify the primary site; (b) to determine if this group of tumors shared unique chromosome anomalies related to transformation and/or progression; and (c) to screen UPT for cytological evidence of gene amplification in the form of double minutes, homogeneously staining regions, differentially staining regions, or abnormally banded regions—findings that could account for their unique and apparently rapid acquisition of the metastatic phenotype.

With regard to our first goal, a review of the cytogenetic properties associated with adenocarcinomas reveals that to date only a handful of these solid tumors have been assigned specific primary chromosome changes (11). This paucity of information regarding primary changes in human solid tumors, in conjunction with the observed karyotypes found in UPT, makes an unequivocable diagnosis of the primary site extremely difficult, if not impossible. Furthermore, from a therapeutic standpoint, identification of the primary tumor site after dissemination to distant organs has occurred is currently of little or no practical value (2).

Of greater interest and possible biological significance is our finding of a number of shared structural anomalies that may be associated with the biological characteristics of UPT, such as invasiveness, metastatic spread, and resistance to therapy. Alterations of chromosome 1, band regions q22-p13, were observed in all four UPT cases (Table 2, Figs. 1-4). These included deletion or nonreciprocal translocation of the short arm, iso-chromosome of the long arm, and evidence for gene amplification. These findings are in general agreement with the abnormalities in chromosome 1 reported by Atkin (9) and others (12-14). In his review of chromosomal aberrations seen in
chromosome 1, Atkin proposed that chromosome 1 changes are most commonly seen in advanced malignancy. Since UPT, by definition, present as metastases, they are “advanced” at the time of their clinical presentation. This is of particular interest in UPT, where a primary tumor is not clinically identifiable.

Since UPT present as advanced disease and contain chromosomal changes consistent with advanced malignancy, we would propose that these tumors undergo an accelerated form of progression. Thus, whereas some tumors (colon, cervix, stomach, melanoma) progress by the classic steps defined by Foulds (3, 4) (type 1 progression), many human tumors (renal, lung, prostate, UPT) do not pass through a stepwise increase in malignancy but are in fact malignant soon after inception (type 2 progression).

The quintessential example of type 2 progression is UPT, for in these tumors the primary tumor either never forms, involutes, or has an extremely slow growth rate. With this premise in mind, the karyotypic changes described in this manuscript may be related to the unique progression of these tumors. It is presumed that UPT metastasize from the primary tumor before they reach a minimally detectable size of 1 cm (10^6 cells, 30 doublings). Thus, the chromosomal changes seen in UPT may bring us somewhat closer to the transformation event, although likely still many steps removed, but closer than metastases from primary tumors of known origin.

The demonstration of karyotypic changes in UPT raises several issues related to a basic view of how tumors progress. We have proposed (15) that the rate of genomic instability in tumor cells is constant rather than increasing as cells become more malignant. This view is supported by our failure to detect differences in spontaneous mutation rates or the rate of generation of karyotypic abnormalities between metastatic and nonmetastatic cells (16, 17). In addition, we would argue that the least successful tumor would be one that becomes more genomically unstable as it acquires an increased malignant phenotype, simply because the chance of a lethal mutation or genomic rearrangement would continually increase. Thus, UPT that by definition are highly malignant (they present as metastases) have already acquired chromosomal changes consistent with advanced disease that may have provided a basis for their malignancy but did not, at the same time, detrimentally affect their survival. These tumors have therefore survived the pitfalls of genomic instability, i.e., they have not been selected “for”; rather, the nonsurviving clones were selected “against” by acquiring random lethal genetic changes. UPT thus provide a model for identifying relevant and irrelevant chromosomal abnormalities, and, as such, they may provide a window into the karyology of neoplasia.

Deletions of the long arm of chromosome 7 were observed in two of four cases (Table 2; Figs. 1-4). Such deletions have not previously been reported as being common in solid tumors. However, deletion or loss of chromosome 7 has been reported in acute nonlymphocytic leukemia (18). This region of chromosome 7 may also contain genes that are important in the tumorigenicty or progression of this group of tumors. It is now reasonably well established that protooncogenes are frequently located at or near the sites of recurring chromosomal changes (19-21). Presumably, the activation or alteration of certain human oncogenes is directly related to tumorigenesis. The MET oncogene has been localized to the 7q22.3-q23.1 region, but the significance, if any, of this to the behavior of UPT is presently unknown.

In summary, our preliminary study of the properties of human UPT has demonstrated that, although these tumors display diverse phenotypic properties in vitro, detailed banding analysis has revealed a nonrandom karyotypic profile which supports our hypothesis that these tumors can be regarded as a neoplastic subgroup. Further studies are currently under way to character-
ize a large panel of UPT to determine if these chromosome anomalies are, in fact, uniformly characteristic of these tumors.

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The authors wish to thank Rosanne Lemon for her secretarial assistance.

Note Added in Proof
Since submission of this manuscript, tumors from an additional four patients with UPT were available for karyotyping. In all four cases 1p\textsuperscript{-} was found along with a number of random karyotypic changes.

REFERENCES
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