

# Substrate dependent genomic heterogeneity in cancers of the lung

## Research Article

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**Abbreviations:** diaminobenzidine, (DAB); double minute, (dm); fetal calf serum, (FCS); geimsa-tripsin-geimsa, (GTG); normal tissue culture plastic, (NTCP); Roswell Park Memorial Institute tissue culture media 1640, (RPMI-1640); variant small cell lung cancer, (v-SCLC)

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## Summary

Split samples from an adenocarcinoma of the lung, an embryonal testicular carcinoma metastasized to lung, and a variant-small cell lung carcinoma (v-SCLC) were cultured on two different plastic substrates, i.e. normal tissue culture plastic (NTCP) and Primaria<sup>TM</sup> flasks. Cells were cultured in identical media. Upon harvesting of the cultures, chromosomal analyses were begun to investigate clonal differences found between the substrates. For each tumor, chromosomal abnormalities were encountered in one plastic, but absent in the other. The greatest differences were noted in v-SCLC. Some cells attached while the others remained suspended in the medium. Both suspension and attached cultures grew. These populations, when subjected to GTG banding or immunohistochemical staining with a panel of eight antibodies demonstrated differences in chromosomal constitution and specific differentiation markers. The universal use of a single combination of substrate and media in tumor cytogenetics may result in an incomplete catalogue of chromosomal anomalies. Classical SCLC is known to evolve rapidly into atypical, chemo- and radiation-resistant SCLC, these changes may reflect the underlying biological progression occurring *in vivo*. We recognize the limited nature of this study and await subsequent studies demonstrating the utility of multiple support substrates in modeling *in vivo* tumor progression. This may offer a starting point for the development of a new diagnostic tool especially for v-SCLC.

## I. Introduction

Cancer cells in general and solid tumors in particular are predominantly multi-clonal. Successful culture of tumor cells is contingent upon the process of cell adhesion. Although normal tissue culture plastic (NTCP) is the gold standard for cell culture, others have modified this system and incorporated or developed new systems to improve cell culture growth. These included agar (Trent and Salmon, 1980), fibronectin (Kleinman et al, 1981; Klebe and Mock, 1982) and ECM's (Siegal et al, 1993).

Malignant ovarian tumors cultured on these same two plastics, i.e. normal tissue culture plastic (NTCP) and Primaria<sup>TM</sup> (Becton and Dickinson Labware, Franklin Lakes, NJ, USA) showed an increased rate of establishment in culture from biopsy material. The success rate was higher than had been shown (Deger, 1997). Presently, we have grown in these same two substrates

(NTCP and Primaria<sup>TM</sup>), an adenocarcinoma of the lung, an embryonal testicular carcinoma metastasized in lung and a variant-small cell carcinoma of the lung (v-SCLC). We analyzed these for genomic differences on the two dissimilar plastic substrates. The highly variable v-SCLC was also examined using a panel of antibodies to differentiation antigens. We investigated whether the differences were associated with corresponding changes in the biology of the cells.

## II. Materials and methods

Tumor materials were aseptically excised, placed in transport serum free RPMI - 1640 in 10 mM Hepes buffered media and transported directly to the laboratory. The tumor tissue was placed in a sterile petri dish in a laminar flow hood where the necrotic and other extraneous material e.g., fat was dissected and removed. The resultant tissue was mechanically disrupted into fine slivers using two sterile scalpels and washed with sterile

media. A minimum amount of media that contained 10% fetal bovine serum (FBS) in RPMI - 1640 fortified with 2% penicillin and streptomycin and supplemented with 2 mM L-glutamine was used to keep the tissue moist. The resultant cell slurry was then overlaid with a solution containing 16mg of collagenase-II in 10 ml of media with 15% FBS in RPMI - 1640 fortified with 2% penicillin and streptomycin and supplemented with 2 mM L-glutamine at 37°C. Disaggregation of the slurry into single cells was monitored by direct visualization by microscopy. The time of incubation varied from 4 hrs to overnight. After the undigested tissue settled, the cells were harvested by centrifugation; washed in RPMI - 1640 fortified with 2% penicillin and streptomycin and supplemented with 2 mM L-glutamine; and incubated at room temperature in RBC lysing buffer (Sigma, St Louis, MO, USA) for 10 min. Cells were washed again, counted, split into the appropriate numbers of flasks. Each culture was plated on Primaria™ and NTCP in media containing 10% fetal bovine serum (FBS) in RPMI - 1640 fortified with 2% penicillin and streptomycin and supplemented with 2 mM L-glutamine.

Cytogenetic analysis was carried out using linear growing, sub-confluent cultures. These cultures were exposed to 0.5 µg/ml colchemid™ for 1-15 hours to increase the number of cells undergoing mitosis; the attached cells were then harvested using 0.06 % trypsin-EDTA. The cells were washed and centrifuged to eliminate the residual trypsin. Suspension cultures were harvested by centrifugation. Cells were exposed to hypotonic sodium citrate solution (1:1 mixture of 0.4% solution containing potassium chloride and sodium citrate). Hypotonic exposure and several steps of harvesting, washing and exposure were carried out by repeated centrifugation and suspension of each of the pellets. This was performed five-times over a twenty-minute period. The cells were then denatured in Carnoy's fixative. Each culture of v-SCLC cells, whether growing in unattached suspended cultures in the media above the plastic flask or attached to the plastic substrate, was initially separated, cultured independently from the line competing cell line and harvested. The fixed and swollen cells were then dropped onto slides in a high humidity environment to both spread and maximize the removal of cytoplasm from the metaphase spreads. Prepared slides were then stained using standard trypsin-geimsa staining method for GTG banding (G-bands obtained by trypsin using Giemsa stain). Comparison of chromosome markers of v-SCLC Primaria™ was obtained using an Olympus microscope system. Approximately 20 cells from each culture were examined and 10 individual cells were scored for chromosomal anomalies by direct examination and photographed. In this manner, clonal lines were then identified and evaluated using ISCN 1995 nomenclature.

Cells were appropriately harvested and prepared for immunohistochemical staining as described by the suppliers. A cytospin preparation of each of the cultures was obtained and the slides air dried and stored at -70°C. Antibodies for CEA, Keratin, NSE, EMA and SCLC specific antibodies TFS2, TFS4 (Okabe et al, 1985) and antibodies MY4 and MY9 (Yamashita et al, 1989) were used in this study. MY4 and MY9 antibodies detected granulocyte macrophage colony-stimulating factor on v-SCLC, as well as leukemic cells. For each experiment, antibody blocking and optimization were performed as described by the manufacturer. In general, frozen slides containing the cells previously concentrated by cytospin centrifugation, were brought to room temperature and prefixed with 3% hydrogen peroxide methanol for 30 min. After a PBS wash, the slides were treated with 1% bovine serum albumin in PBS for 30 min followed by an exposure of 1:20 dilution of normal serum albumin in PBS for 30 min followed by, for example, 1:100 rabbit antihuman keratin (primary antibody) in PBS for 30 min and a 10min wash. The slide was then exposed to PAP (1:50 in PBS) for 45 min and a PBS wash. Diaminobenzidine was prepared as follows: first a

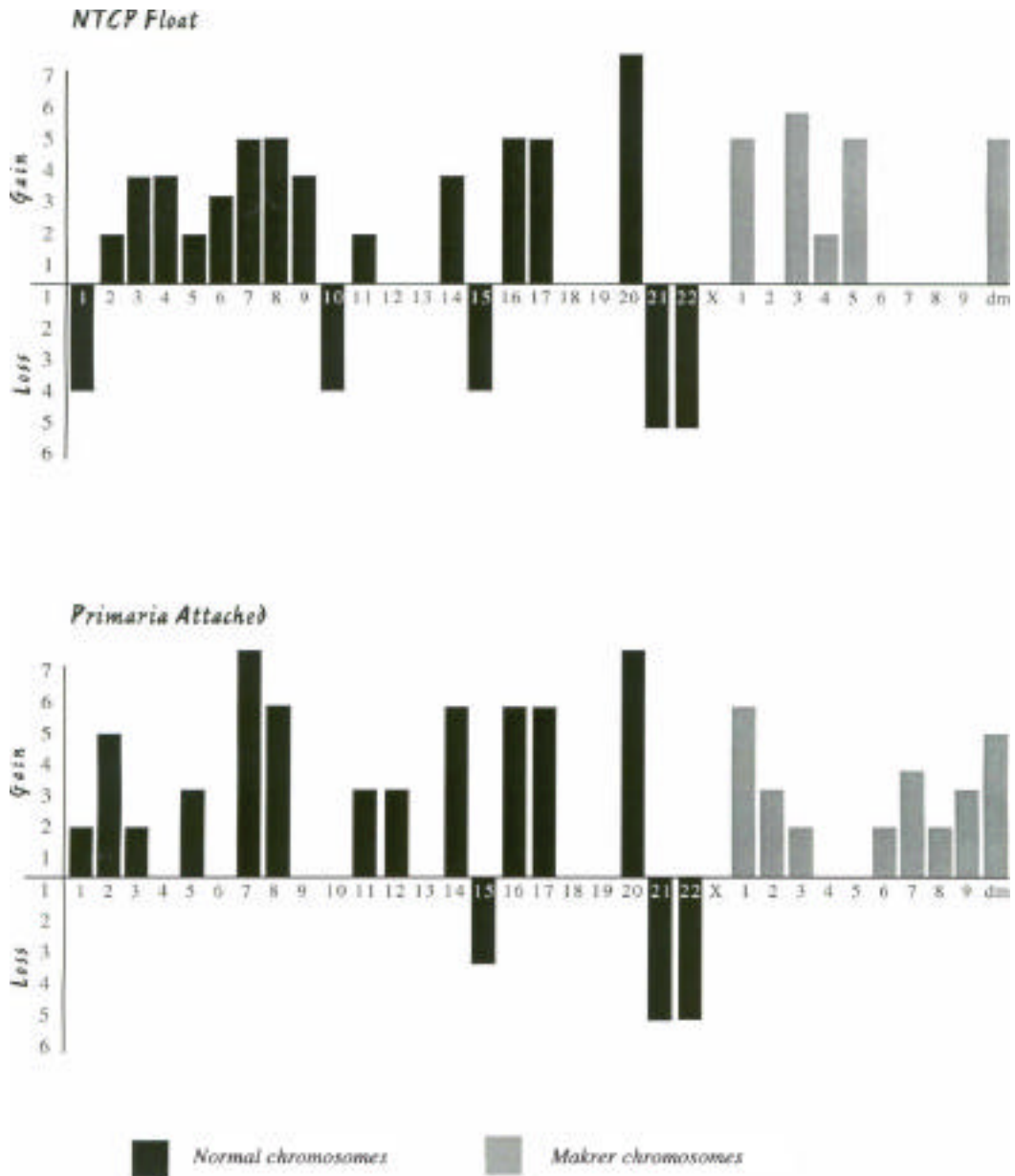
solution was made by mixing 10 ml of 0.5 M Tris-HCl buffer to 90 ml of dH<sub>2</sub>O from which 12 ml of solution was discarded. A second solution of 1 ml 30% H<sub>2</sub>O<sub>2</sub> was added to 90 ml dH<sub>2</sub>O. Finally a third mixture was made by mixing 0.75ml of each of the three solutions with the final addition of 0.11gm of diaminobenzidine. Stain was filtered and each slide was stained for 5 min. The slides stained for antibodies CEA, NSE and EMA were processed the same way as the slide for keratin. For TFS2 prior to the exposure to primary antibody, the slide was exposed for 8 min in 10% normal goat serum and then exposed to 1:100 monoclonal mouse antihuman TFS2 for an additional 40 min. After a 10 min PBS wash, the slide was exposed to goat anti-mouse-biotin, the secondary antibody. A PBS wash was followed by exposure to ABC complex for 30 min. DAB staining was the same as explained earlier. The staining procedure for TFS4, MY4 and MY9 was the same as explained for TFS2. Slides were counter-stained with toluidine blue. Reaction to the cell by the antibody was graded both based on intensity (graded from 1-3), as well as percentage of the stained cells.

### III. Results

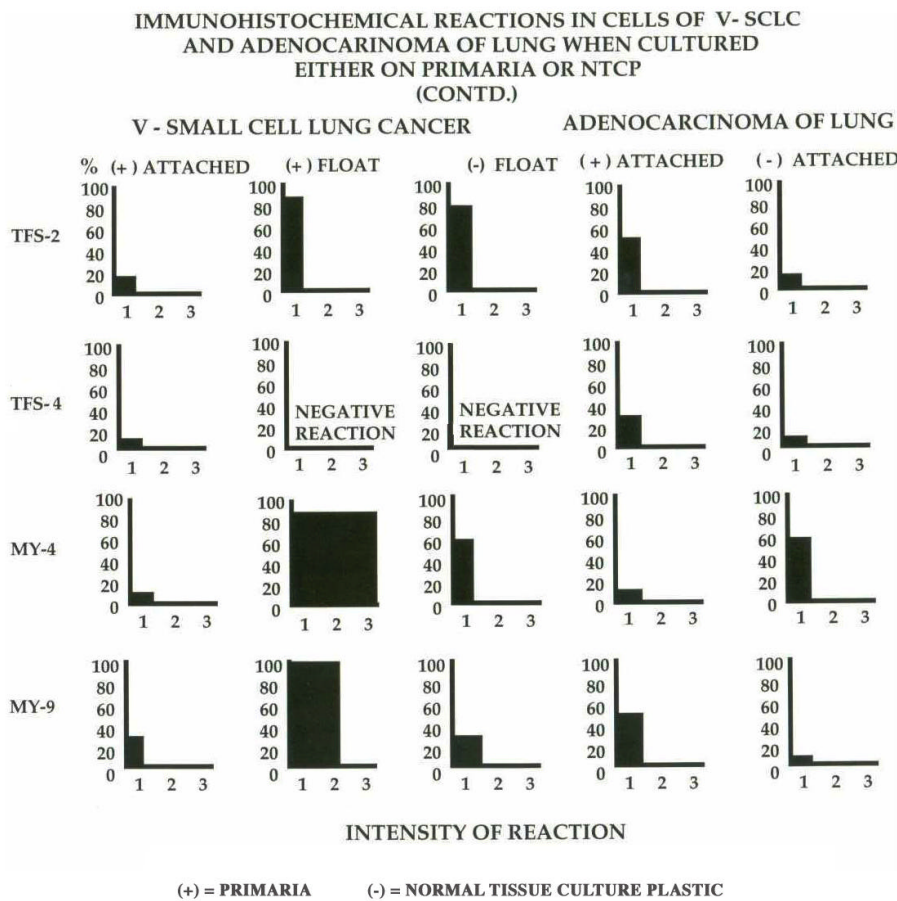
Tumor biopsies were brought into the laboratory, processed and cultured as described. Cellular harvests of the cultures enriched for mitotic cells were accomplished using standard techniques. Ten cells from each substrate were analyzed. This included analyzing cells that were attached to the plastic (stickers) and those cells that remained growing in suspension (floaters). The suspension cells were separated from the attached cultures and grown separately. The suspension cultures did not attach even after longer periods of growth.

In the adenocarcinoma of the lung grown on NTCP, two abnormal clones, i.e. one showing 45 chromosomes with the loss of the Y chromosome was found in three cells (45,X,-Y[3]) and the other clone showed two cells with 47 chromosomes with the addition of a marker chromosome (47,XY,+mar[2]). These two abnormal tumor clones were found in addition to the normal karyotype that was found in five cells (46,XY[5]). On Primaria™, a normal clone of five cells (46,XY[5]) was also found with only a single abnormal clone 45,X,-Y[4].

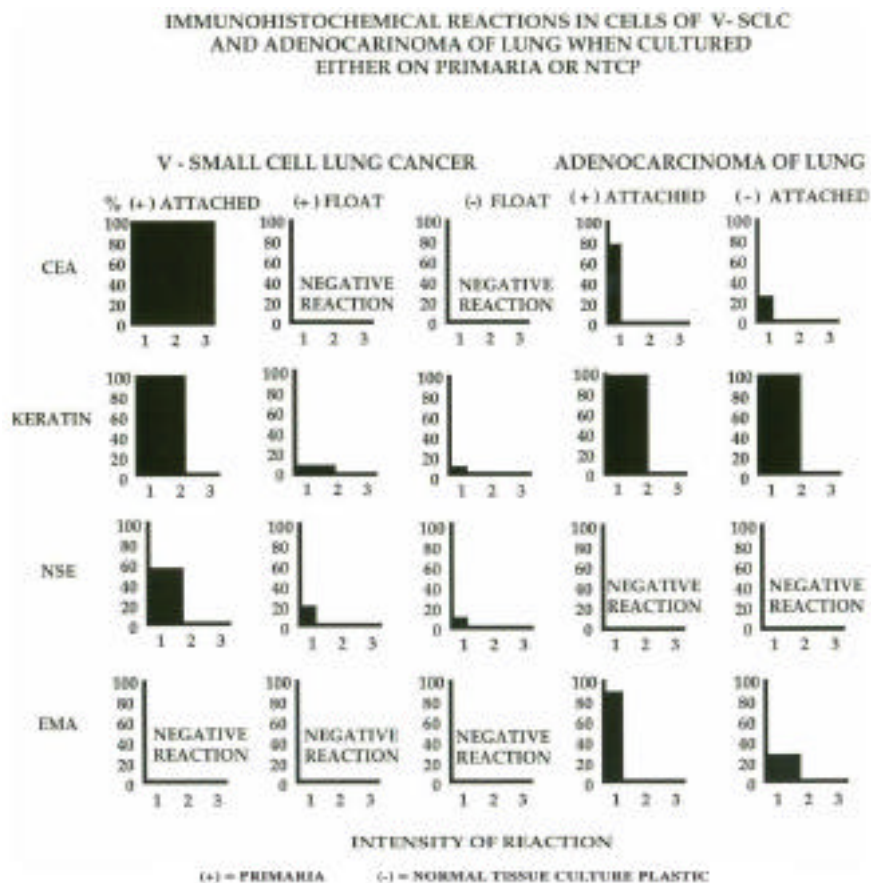
In addition to chromosomal variation found in the adenocarcinoma, NTCP and Primaria™ showed distinct culture properties. The variant SCLC tumor differed in the ability to adhere to the two plastics. The tumor cells in NTCP did not attach to the plastic, but remained floating in the media. Nonetheless, these cells continued to grow. Cells cultured on Primaria™ showed two distinct populations. The first, the cells remained suspended in the medium while remaining active. The other, as expected, attached to the surface of the flask. Chromosomes of NTCP ranged from hypodiploid to hypotriploid with a hypotriploid mode. In Primaria™ however, the attached cells ranged from hyperdiploid to hypertriploid. However, chromosomal distribution showed hypertriploidy as the most common outcome in both plastics. Strikingly, cells growing in suspension on Primaria™ showed a single abnormal clone, 45XX,-16, (see **Table 1**). The attached cell population cultured on Primaria™ and cells suspended in the medium contained in NTCP showed 24 chromosomal anomalies each. Sixteen of the twenty-four were common while, eight were unique clones (**Figure 1**).



**Figure 1. Chromosomal analysis of v-SCLC biopsies split and established on different tissue culture plastics.** Disaggregated cells were split and established. The resultant cells were cultured on normal tissue culture plastic (NTCP) or Primaria™. Abnormal chromosome numbers are plotted as black bars above the axis representing chromosome gains and below the axis representing chromosomal losses. The grey bars represent the presence of structurally modified chromosomes called markers.



**Figure 2.** Immunohistochemical evaluation in v-SCLC cultures and adenocarcinoma of the lung cultures grown on normal tissue culture plastic or Primaria™. Each of the cell lines established under the different conditions were reacted to each of the antibodies as described. Immunohistochemical reactions were graded and the scores represented by the height of the vertical bars (percentage of cells reacted positively), while the width of bars represents the cellular intensity of the staining process.



**Figure 3.** Immunohistochemical analyses of v-SCLC and adenocarcinoma of the lung grown on normal tissue culture plastic or Primaria™. Immunohistochemical reactions were graded and the scores represented by the height of the vertical bars (percentage of cells reacted positively), while the width of bars represents the cellular intensity of the staining process.

To further investigate the biological impact of these differences the cultures were characterized for expression levels of eight specific differentiation markers. Each of the three cultures was immunohistochemically stained for each marker and the intensity scored on a scale (0-5). The attached cells to Primaria™ showed intense reaction to CEA and keratin in 100% of the cells. NSE staining was also positive in 50% of the cells. Conversely, the cells suspended in the medium grown in NTCP and Primaria™ showed no reaction to CEA and little or no staining to keratin and NSE. EMA staining was absent in all the populations studied (**Figure 2 and 3**).

Three of the monoclonal antibodies specific to SCLC showed reaction to the cells suspended in medium when cultured in the Primaria™ flasks. The reactions of MY4 and MY9 antibodies showed moderately and highly intense staining, respectively. In cells cultured on NTCP however, only a very sparse number of cells showed any reaction to these same monoclonal antibodies. None of the scored cells showed an intense staining reaction (**Figure 3**). Original biopsy cells recovered by culturing on these two chemically diverse tissue culture plastics not only showed chromosomal clonal differences, but these differences were mirrored by expression of specific differentiation antigens. This demonstrated that cells in these two plastics did not only differ in their genome but also biologically. The cell populations from the two substrates of adenocarcinoma of the lung showed similar reaction to the antibodies.

#### IV. Discussion

Cytogenetic studies of cells grown on surfaces other than normal tissue culture plastic started more than a decade ago (Trent and Salmon, 1980; Roberts and

Tattersall, 1987; Crickard et al, 1989; Satyaswarup and Tabibzadeh, 1991). Differential growth of cells on different substrates was previously documented. Chemically or spatially distinct substrates can interfere with the biology of cells in varied ways (Westphal et al, 1990; Vadlamuri et al, 2003). Specific examples of mutated genes also interfered with cell adhesion (Hesketh 1994). To our knowledge, a comparative study with respect to the biology or cytogenetics of the same tumor derived from cultures on normal and modified surfaces has never been described. Neither of these culture methods were previously used to evaluate the genetic status or evolution of neoplastic diseases.

In the present study, we find differences in the clonal distribution of cells of lung cancers when simultaneous split cultures were established on either NTCP or Primaria™ (**Figure 1**). For example, in adenocarcinoma of the lung, clones obtained from cells cultured on NTCP showed two unique markers while only one was recovered from cells grown on Primaria™. In paradox, v-SCLC demonstrated greater heterogeneity within the Primaria™ cell population when compared to NTCP clones. On Primaria™ two kinds of cell populations were recovered. One attached to the plastic while the other remained in suspension in the media. For NCTP, only cells suspended in the media were found. The two populations of Primaria™ differed from each other in their genomic constitution and in their immunohistochemical responses (**Table 1, Figure 1-3**).

Primaria™ floating cells were either diploid or hypodiploid, while the stickers were hypotriploid with a total of 23 chromosomal anomalies. Although the cells of NTCP were hypotriploid and had 23 chromosomal anomalies, the two populations differed from each other by eight unique chromosomal abnormalities.

**Table 1.** Karyotypic differences in three tumors of the lung when grown either on Normal Tissue Culture plastic (NTCP) or Primaria™

Tumor Type	Substrate	Karyotype
Adenocarcinoma of the lung	NTCP	45,X,-Y[3]/47,XY,+mar[2]/46, XY[5]
Adenocarcinoma of the lung	Primaria	45X,-Y[4]/46,XY[5]
Testicular germ-cell tumor from the lung	NTCP	46,XY,+6mar[5]/46,XY+mar[2]/46,XY[4]
Testicular germ-cell tumor from the lung	Primaria	46,XY,2mar[2]/46,XY[7]
v-SCLC floating cells	NTCP	44-46,X/XX,-1,+2,+3,+4,+5,+6,+7,+8,+9,+10,+14,-15,+16,+17,+20,-21,-22,+4mar,dmin[cp11]
v-SCLC attached cells	Primaria	48-65,X/XX,+1,+2,+3,+5,+7,+8,+11,+12,-14,-15,+16,+17,+20,-21,-22,,+7mar,dmin[cp7]
v-SCLC floating cells	Primaria	45,XX,-16{3}/46,XX[5]

Immunological results further support differences between these two populations (**Figure 2 and 3**). It is of note that similar reactivity to all the antibodies tested was demonstrated between the unattached, floating cell populations from Primaria™ and those from NTCP. Genomic differences between the two floaters were far greater than the differences between the NTCP floaters and Primaria™ stickers. Genomic and differences in biology of v-SCLC point out that the tumor is heterogeneous demonstrating distinct clones. Distinguishing clones were not found in either of the other two lung malignancies.

Small cell lung cancer progresses into a chemo- and radiation resistant variant with altered prognosis (Leij et al, 1985; Bepler et al, 1987). There are unbiased approaches to investigating genetic and chromosomal quantitative changes. Pioneered by comparative genomic hybridization, new genome complete and high resolution contigs on microarrays exist as well as the newer application of oligo microarrays for chromosomal analysis. These techniques do not have the requirement for growing cells, but may be biased both by the purity of the original sample as well as by the presence of DNA isolated from non-mitogenically active tumor cells.

CGH have also shown differences between SCLC and atypical-SCLC. Using both NTCP and Primaria™ substrates to culture v-SCLC, we were able to recognize cell populations with different genomes and biology. These findings will need future study to clarify the significance and mechanisms of the difference found. It is encouraging that the chromosomal differences were mirrored by the expression of specific differentiation antigens. This culture technique in combination of techniques such as CGH and FISH (Ashman et al, 2002; Johnen et al, 2003) may provide new insights into the initiation and progression of this high mortality cancer. In the future, multiple techniques will provide new tools for studying the etiology and evolution of classical SCLC into v-SCLC.

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