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ARTICLE

# Establishment and Characterization of a Human Large Cell Lung Cancer Cell Line with Neuroendocrine Differentiation

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**Characterization of a human lung cancer cell line is reported. This cell line was established from a patient referred to Nemazi Hospital of Shiraz University of Medical Sciences with a diagnosis of poorly differentiated carcinoma. Sterile sample from peritoneal effusion was taken and immediately cultured in RPMI-1640 medium containing 20% FBS, at 37C with 5% $CO_2$. This cell line has been in continuous culture for more than one year and has been named as Mehr-80. Several features of the cell line were investigated, including growth characteristics, electron microscopic features, cloning efficiency in soft agar, expression of various antigenic markers, chromosomal and DNA analysis. On the basis of morphological and immunohistochemical analysis of Mehr-80, it is possible to conclude that this cell line is characterized by features similar to those reported for large cell carcinoma with neuroendocrine differentiation (LCCND). This cell line will be a valuable *in vitro* tool for further studies on lung cancers.** (Pathology Oncology Research Vol 10, No 4, 225–230)

*Keywords:* cell line, lung cancer, large cell carcinoma, neuroendocrine differentiation

## *Introduction*

Lung cancer is currently the most frequently diagnosed cancer and the most common cause of cancer mortality in males worldwide.[1,2] The general classification of lung tumors is non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). However, there are lung tumors that cannot be classified using these categories. Revised classification of lung and pleural tumors was carried out by a WHO selected committee in 1994. Later in 1999 the committee reclassified large cell carcinoma of the lung into four types.[3] The first type is large cell neuroendocrine carcinoma (LCNEC), which is represented as cells having neuroendocrine morphology and expressing features of neuroendocrine differentiation by immunohistochemistry and/or electron microscopy. The second type is large cell carcinoma with neuroendocrine differentiation (LCCND), which is represented as cells lacking neuroendocrine morphology but having neuroendocrine markers by immunohistochemistry or electron microscopy. The third type is large cell carcinoma with neuroendocrine morphology (LCCNM), which has neuroendocrine morphological features but lacks neuroendocrine markers by immunohistochemistry or electron microscopy, and finally classical large cell carcinoma, which lacks neuroendocrine morphology or differentiation features.[4] In recent years the classification of lung tumors and lung cancer cell lines has been investigated by gene expression profiling, which has provided evidence for more heterogeneity in lung tumors.[5-8]

Reports on establishing of large cell lung cancer cell lines with heterogeneity in morphology and cell biology are still progressing,[9-12] which is exemplified by the establishment of large cell lung cancer cell lines producing high amount of G-CSF.[11,13-16] In this study we established a lung cancer cell line with aggressive behavior. The first diagnosis of this tumor was poorly differentiated carcinoma, but after further morphological, immunohistochemical and electron microscopic studies it was apparent that this cell line has most features of large cell carcinoma with neuroendocrine differentiation.

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***Patient and establishment of cell line***

Mehr-80 cell line was derived from the peritoneal fluid of a 39-year-old female. She had seen the physician because of hemoptysis and cough. Mediastinal CT showed large isodense extrapleural mass around the left second rib. Bronchial lavage was negative. In abdominal sonography there were two masses in the right and left lobe of the liver, and multiple abdominal metastatic lesions. Biopsy of the masses was performed and histologic diagnosis was high-grade sarcoma or poorly differentiated carcinoma. The patient was operated on, and the masses which had extended into the chest wall were excised. The final pathologic diagnosis on tissue sections was poorly differentiated carcinoma. The patient died one month after surgery due to widespread organ metastasis.

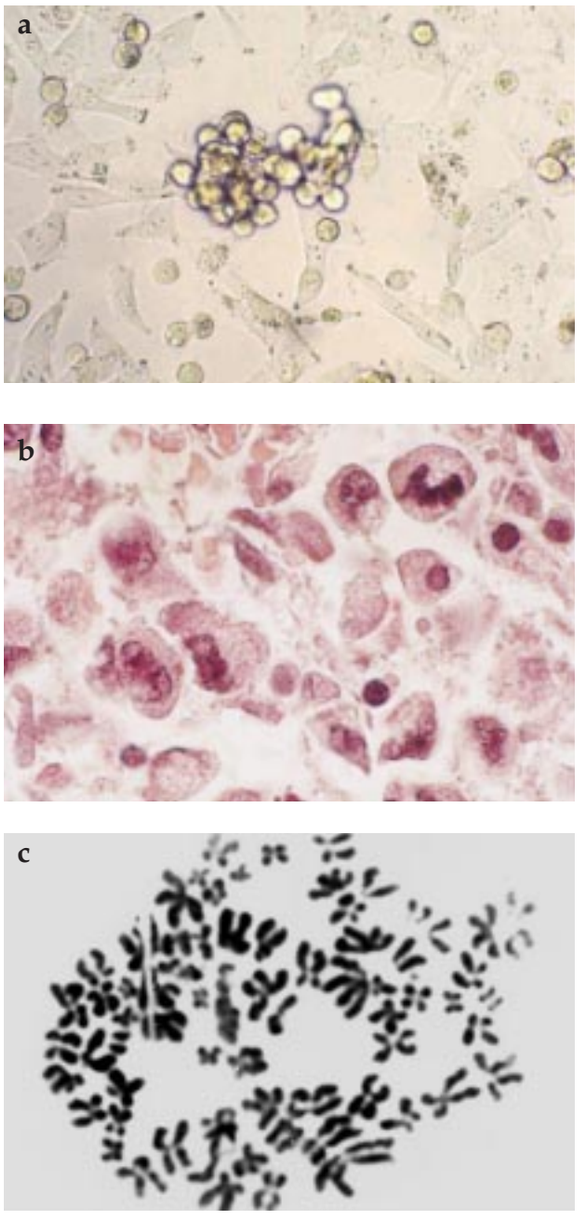


***Figure 1 (a)*** *Morphological features of cultured Mehr-80 cells by phase contrast microscopy (x200).* ***(b)*** *Histology of the original tumor showing numerous malignant large cells with abundant eosinophilic cytoplasm, marked nuclear pleomorphism and irregular nuclear border, with mitotic features (x400, HE).* ***(c)*** *The chromosomal feature of a tumor cell.*

Sterile sample was obtained from peritoneal effusion. This fluid was layered over a Ficoll-Hypaque density gradient, and the interphase cells were collected. The resulting cell suspension was washed, resuspended in RPMI-1640 with 20% FBS (Gibco) and 2 mM glutamine, 100 ìg/ml streptomycin and 100 IU penicillin. Cells were placed in 25 $cm^2$ culture flasks and kept at 37°C in a humidified atmosphere with 5% $CO_2$. After incubation for a few days, cells grew as firm adherent monolayer with some floating cells. Growing cells were fed twice weekly by the above culture media with 10% FBS, and subcultured once a week by trypsinization.

*Growth characteristics*

To determine population doubling time, single-cell suspensions were prepared after trypsinization of the culture and drawing the cells up and down several times through a 21-gauge needle. The cells were plated in triplicate at a concentration of $2x10^4$ cells/ml in 24-well culture plates. Cell counts were performed every day for one week. Doubling time was determined from the slope of the growth curve during exponential growth.

*Cloning efficiency in soft agar*

Bottom layers of 0.5% agarose with 20% FBS in RPMI-1640 were allowed to harden in 35 mm plastic dishes. Single-cell suspensions of the cell line in 0.3% agarose with RPMI-1640 + 20% FBS were poured over the agarose layers. Cell concentrations of 100 to 1000 cells per plate were tested. Colonies with more than 50 cells were scored after 14 to 21 days. The colony-forming efficiency was determined as the number of colonies per number of tumor cells, multiplied by 100.

*Immunohistochemistry*

Immunoperoxidase staining was performed both on formalin-fixed tissue sections and also on cells prepared from the growing cultured cell lines. A standard technique was used to determine the presence of a number of antigens, using immunoperoxidase reactions for immunohistochemistry. Antibodies to cytokeratin, vimentin, desmin, actin, cytokeratin 5/6, NSE, chromogranin A, S100, surfactant

***Table 1.*** **Immunohistochemistry results on cell line and tissue section**

| | |
|---|---|
| *Tissue section* | |
| Cytokeratin | Diffuse strongly positive |
| Vimentin | Focal moderately positive |
| Desmin | Negative |
| LCA | Negative |
| Synaptophysin | Weakly positive |
| Chromogranin A | Positive |
| *Cell line* | |
| Cytokeratin | Positive |
| Vimentin | Positive |
| Desmin | Negative |
| Actin | Negative |
| NF | Negative |
| Cytokeratin 5/6 | Negative |
| Chromogranin A | Positive |
| S100 | Negative |
| NSE | Negative |
| Synaptophysin | Weakly positive |
| Surfactant protein A | Negative |
| HMB45 | Negative |
| PI-ALK | Negative |
| EGFR (HER-1) | Positive |
| HER-2 | Negative |
| p53 | Negative |

protein A, CEA, HMB45, EGFR, HER-2, p53, LCA, NF, PI-ALK, and synaptophysin were used. All antibodies and reagents were obtained from Dako (Denmark).

*Chromosome analysis*

Cell suspensions from cultured cells were incubated in fresh culture medium for 24 hours, and then with colcemide (Sigma, USA) at a final concentration of 0.04 ìg/ml for 2.5 hours. The cells were suspended in hypotonic potassium chloride solution (0.075 M) for 15 minutes, fixed in 3:1 methanol / glacial acetic acid, and spread on cold glass slides. Slides were dried at 57°C before staining with Giemsa for examination.

*DNA index*

The CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson, USA) was used for isolating and staining cell nuclei from tumor cell suspensions. The method involves dissolving the cell membrane lipids with a nonionic detergent, eliminating the cytoskeleton and nuclear proteins with trypsin, digesting cellular RNA with RNase, and stabilizing the nuclear chromatin with spermine. Propidium iodide was added to the cell suspension to stain nuclei. The cells were then run through the FACScalibur (Becton Dickinson) for estimating DNA index (DI) and cell-cycle phase distributions.

*Electron microscopy*

Cells were centrifuged and the sediment was fixed in 3% glutaraldehyde, buffered with 0.2 M cacodylate, at 4°C, postfixed in 1% osmium tetroxide, dehydrated through ascending series of ethanol, and embedded in Epon 812. Semithin sections were cut and stained with toluidine blue, and were used for light microscopic orientation. Ultrathin sections were cut from selected areas, mounted on copper grids and stained with uranyl acetate and lead citrate, then examined under LEO 906 electron microscope.

## *Results*

*Morphological features of the cultured Mehr-80 cells*

Mehr-80 cells adhered firmly to the culture dish. The adherent cells were morphologically polygonal and epithelial-like, containing intracytoplasmic granules. Morpho-

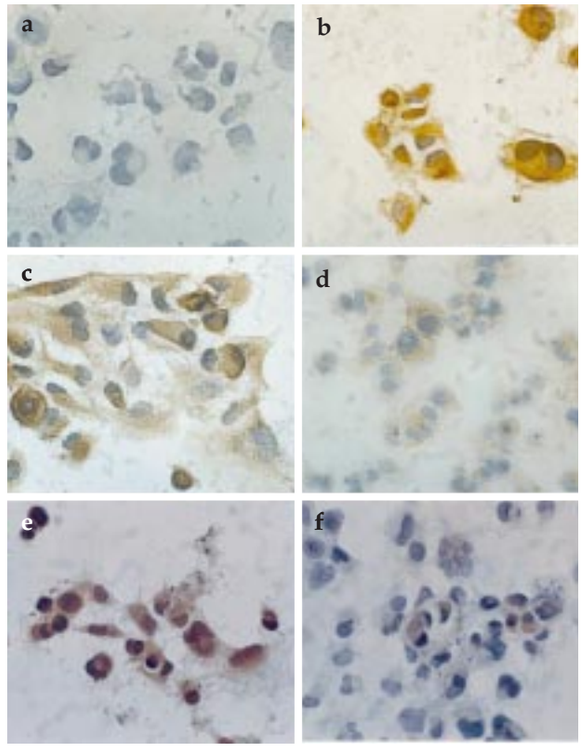


***Figure 2.*** *Immunostaining of the cell line.* ***(a)*** *Negative control.* ***(b)*** *Cytoplasmic immunoreactivity for cytokeratin.* ***(c)*** *Positive staining for vimentin.* ***(d)*** *Positive staining for EGFR.* ***(e)*** *Positive staining for chromogranin A.* ***(f)*** *Weakly positive staining for synaptophysin. (x400).*

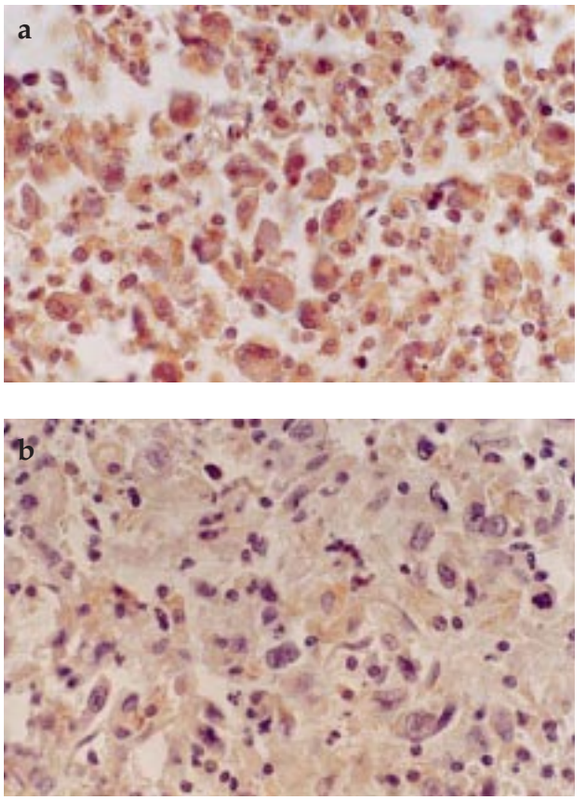


***Figure 3.*** *Immunoreactivity of the original tumor for neuroendocrine markers.* ***(a)*** *Positive staining for chromogranin A.* ***(b)*** *Weakly positive staining for synaptophysin (x400).*

logical features of cultured Mehr-80 cells by phase contrast microscopy is demonstrated in *Figure 1a*. Histology of the original tumor was investigated by HE staining. As shown in *Figure 1b,* numerous malignant large cells with abundant eosinophilic cytoplasm, marked nuclear pleomorphism and irregular nuclear border with mitotic features are revealed.

*Growth characteristics*

The doubling time for this cell line was calculated from the slope of the growth curve, and was 29 hours. In addition, cloning efficiency of Mehr-80 cells was investigated on soft agar and was found to be 2.8%.

*Chromosome analysis*

Cytogenetic analysis of Mehr-80 cells was performed by routine karyotyping. Examination of metaphase spread was performed. The chromosome number varied between 46 and 92. Most of the karyotyped cells had polyploid characteristics (*Figure 1c*).

*DNA index*

The DNA index (DI) was defined as the ratio of the peak channel number of the G1/G0 tumor cell population divided by the peak channel number of G1/G0 diploid reference population. The histogram of DNA content of this cell line was analyzed. There were three peaks, one diploid and two aneuploid. The diploid population was 40.65%, while the two aneuploid ones were 52.45% and 6.9%, with DNA indices of 2.37 and 4.59, respectively (data not shown).

*Immunohistochemistry*

Mehr-80 cells were analyzed for expression of a panel of cell surface markers by indirect immunostaining. Results of immunoperoxidase reactions on cultured cells and tissue sections for various markers are listed in *Table 1*. As demonstrated, Mehr-80 was positive for cytoplasmic immunoreactivity of cytokeratin (*Figure 2b*). Moreover, Mehr-80 was positive for expression of vimentin (*Figure 2c*). In addition, a positive staining for chromogranin A was detected both on tumor section and on the cultured cell line (*Figures 3a and 2e*). A weak staining for synaptophysin on the original tissue section and the cultured cell line was shown (*Figures 3b and 2f*). Mehr-80 cell line was also labeled for the expression of EGFR by immunoperoxidase, and found positive for membrane expression of EGFR (*Figure 2d*).

*Electron microscopy*

Semithin sections of cultured cells stained by toluidine blue, as well as ultrathin sections studied under electron microscope revealed isolated large tumor cells with abundant cytoplasm showing prominent rough endoplasmic reticulum and scattered ovoid mitochondria as well as multiple vacuoles of different sizes (*Figure 4A-F*). The cell membrane was demonstrated to have multiple short microvilli measuring about 1 ìm in length (*Figure 4C,D*). The nucleus showed one to two prominent nucleoli, and multiple invaginations of cytoplasm into the nucleus, resembling a nucleus with multiple branching parts (*Figure 4A,B*). Nuclei of some of the tumor cells showed dense perinuclear membrane condensation of chromatin (*Figure 4E*). No definite electron-dense neurosecretory granules were identified in the cytoplasm of tumor cells.

## *Discussion*

In this study we established a cell line (Mehr-80) from peritoneal effusion of a patient with large cell undifferentiated carcinoma of the lung. We used a panel of antibodies to investigate the nature of tumor cells. Immunohistochemistry showed positive staining for both cytokeratin

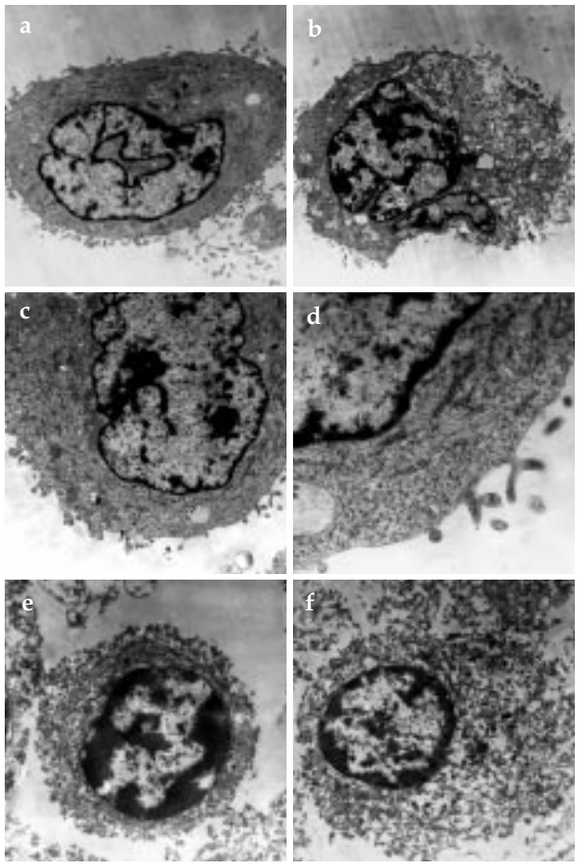


***Figure 4.*** *Electron microscopy of tumor cells from the cell line* ***(a-e).*** ***(a)*** *Prominent rough endoplasmic reticulum and scattered ovoid mitochondria (x4646).* ***(b)*** *Cytoplasmic vacuoles and cytoplasmic invaginations into the nucleus (x3460).* ***(c,d)*** *Multiple microvilli with secondary branching (x5285, x16,700).* ***(e)*** *Perinuclear membrane condensation of chromatin (x10,000).* ***(f)*** *Electron microscopy of a tumor cell from paraffin-embedded tissue biopsy, showing multiple microvilli (x7750).*

and vimentin. Although cytokeratin in most cases specifies the epithelial origin of cells while vimentin is regarded as an indication of mesenchymal origin, co-expression of these two markers in certain carcinomas, like poorly differentiated adenocarcinoma and large cell carcinoma of the lung, has previously been reported.[10,17]

We also observed positive staining for chromogranin A and weak positivity for synaptophysin. Expression of these markers is indicative of neuroendocrine differentiation of this tumor, but with electron microscopy neurosecretory granules (dense core vesicles) were not observed, and the morphology of the tumor was not exactly compatible with the criteria set up for classical large cell carcinoma with neuroendocrine morphology.[9] The chromosomal studies showed a range of 48 to 92 chromosomes in this cell line. This is in agreement with the result of DNA index analysis showing two aneuploid peaks, indicating that the second aneuploid peak consisted of a tetraploid population. The DNA index was relatively high and, as some authors have suggested, the value of DI in ADC (abnormal DNA content) cases may be used to predict prognosis.[20-22] The doubling time of our cell line was found to be 29 hours and cells grew fast in culture without difficulty.

In conclusion, data of our analysis indicate that the features of the Mehr-80 cell line are compatible with a rare malignant tumor of lung, the so-called large cell carcinoma with neuroendocrine differentiation (LCCND), which lacks neuroendocrine morphology but has neuroendocrine markers by immunohistochemistry.[4]

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