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Original Paper

Chromosomal Alterations, Biological Features and *In Vitro* Chemosensitivity of SCLC-R1, a New Cell Line from Human Metastatic Small Cell Lung Carcinoma

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A new human cancer cell line was established from a metastatic lesion of a small cell lung carcinoma (SCLC-R1) and maintained in continuous culture with a doubling time of 62 h. The SCLC-R1 line, whose ultrastructural features are presented, showed a diploid DNA content, a translocation involving chromosome 16 [t(16;?)(q24;?)] and noticeable deletions in the *FHIT* (fragile histidine triad) region in the short arm of chromosome 3 [del(3)(p14)] and in the telomeric region of the short arm of chromosome 12 [del(12)(p13)]. The involvement of 12p in metastatic small cell lung cancer is reported here for the first time. No amplification or rearrangements were evident in the c-*myc*, L-*myc*, N-*myc*, *int-2*, c-*erbB-2*, H-*ras*, K-*ras*, c-*mos*, and *hst-1* genes by Southern blot analysis. Wild-type *p53*, *RB*, K-*ras* and H-*ras* genes were evident by polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis. The neuron specific enolase (NSE) level was much higher in the cell line's cytosol than in the patient's serum and the cell line also had high expression of chromogranin A and cytokeratin 19. SCLC-R1 cells were sensitive to cisplatin, carboplatin and doxorubicin. The clinical history of the patient from whom the cell line was derived is reported. The characteristics of this new cell line indicate it to be a useful experimental model to investigate lung cancer biology and anticancer drug response. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: small cell lung carcinoma, cell line establishment, karyotype, chemosensitivity, gene amplification, gene mutation, tumour marker, DNA index, ultrastructural features, patient's clinical history

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INTRODUCTION

LUNG CANCER is the leading cause of cancer death, with mortality approaching 90% and a median survival of approximately 12 months [1]. Its incidence remains high in the industrialised world and is rapidly increasing in developing countries, due to the strong association with cigarette smoking [2].

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The most common histological classification, proposed by the World Health Organization [3], divides lung tumours into four major categories, representing 95% of all lung cancers and among which 20–25% is due to small cell carcinoma. From a clinical point of view, bronchiogenic tumours are categorised into two groups, which reflect their biological behaviour and systemic or local therapeutic management: small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC), including adenocarcinomas, squamous cell and large cell carcinomas. The interface between the two

groups needs frequent revision due to the advances in knowledge of their cellular and molecular biology [4].

It is known that both inherited predisposition and acquired somatic genetic changes contribute to lung tumour development and progression [5]. It has been postulated that such genetic events may be related to the resistance to chemotherapy, either intrinsic or acquired, which represents one of the major obstacles preventing cure in patients affected by SCLC [6]. The availability of well characterised lung tumour cell lines, derived directly from patients with a documented clinical history and retaining properties observed *in vivo*, provides fundamental models for genetic and biological studies and for *in vitro* search of more effective treatments [7].

We report here the establishment and characterisation of a new cell line derived from a metastatic small cell carcinoma of the lung (SCLC-R1). The clinical history of the patient from whom the cells were derived is presented, together with the ultrastructural, cytogenetic and molecular features of the cells. The results of tumour markers, immunohistochemistry and chemosensitivity assays are shown in comparison with those evident in the patient.

MATERIALS AND METHODS

Clinical findings

The cell line was obtained from a metastatic supraclavear lymph node of a 65 year old man affected by SCLC (stage III). The patient had been treated with endoxan, 4′-epidoxorubicin and etoposide, with a response higher than 80%. Surgical resection of the lung was then performed. Three months later, metastatic supraclavear lymph nodes were detected (cell line derived from one of these) and a new treatment (cisplatin plus etoposide) was started. Eight months later the patient died of disseminated, metastatic disease. The patient was a heavy smoker.

Cell culture and growth

The tumour sample was freed from the stromal elements by 2 h incubation at 37°C with collagenase (1000 units/ml) and hyaluronidase (500 units/ml). The resulting clumps of tumour cells were collected by filtration (50 μm pore size) and then plated in plastic flasks.

The cell line was kept and cultured in a medium containing equal parts of Dulbecco's modified Eagle's medium (DMEM) and Ham's media supplemented with 10% fetal calf serum (FCS), 1% L-glutamine and 1% insulin. The medium was routinely changed every 5 days. At confluence the cells were subcultured by exposure to trypsin (0.05% trypsin with 0.1% ethylene diamine tetraacetic acid (EDTA) in a Ca^{++} and Mg^{++} salt free solution). The 33285 Hoechst stain was routinely employed for mycoplasma testing. Cell growth was monitored by phase contrast observation.

Growth curves were prepared by using cultures in triplicate in 35 mm plastic dishes. Cells (5×10^6) at the 20th and 40th passages of the cell line were plated and the number of viable cells determined in a haemocytometre every 24 h for 10 days by Trypan Blue staining. The doubling time was calculated during the exponential growth phase of the cells. The cells were maintained in culture for up to 100 passages.

Morphology and ultrastructural features

The cell samples were fixed in methanol, stained with haematoxylin/eosin and examined with a light microscope. For electron microscopy analysis, the subconfluent glass-adherent SCLC-R1 cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 30 min at 4°C and post-fixed in 1% osmium tetroxide. After dehydration in ethanol, the samples were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. The same monolayers were re-embedded and sectioned perpendicularly.

Cytogenetic analysis

Metaphase spreads were prepared by standard procedures [8] from the human lung cancer cells of the SCLC-R1 line passaged 20 times. Cytogenetic analysis was performed by QFQ banding [9] and GTG banding [10].

Fluorescent in situ *hybridisation (FISH)*

Metaphases were prepared according to the conventional cytogenetic techniques after colcemide treatment, from SCLC-R1 cells at passage 20 and PHA-stimulated lymphocyte nuclei. Two probes, one specific for the centromeric region of chromosome 12 (18G6) and the second mapping 12p13.1-3 and containing the sequence of the nucleolar protein NOL1 (147G1) were combined [11]. The probes were labelled by nick translation, 18G6 by digoxigenin-11-dUTP and 147G1 by biotin-16-dUPT, according to the instructions of the supplier (Boehringer-Mannheim, Germany). Pretreatment and denaturation of the slides were performed as previously described [21]. Ten microlitres of hybridisation mixture (50% formamide, 2×SSC, 1 μg/ml salmon sperm DNA, 10% dextran sulphate) containing 10 ng/μl of each probe and 200 ng/μl Cot-1 DNA were denatured for 10 min at 100°C, allowed to re-anneal for 30 min at 37°C and applied to the denatured cells. Hybridisation was performed overnight at 37°C in a moist chamber. The slides were washed at 37°C in 2×SSC pH 7 (three times for 5 min each) and 0.1×SSC (three times for 3 min and once for 10 min). Immunocytochemical detection was performed as described previously [12]. Nuclei were stained by 4′, 6-diamidine-2′-phenylindole (DAPI; Sigma, Aldrich, Italy) in antifade. Microscopic evaluation was performed using a Leitz Microscope equipped with filters for fluorescein isothyocyanate (FITC), tetrarhodamine isothyocyanate (TRITC) and DAPI.

Southern blot analysis

High molecular weight DNA was prepared from the SCLC-R1 cells and from normal tissue by standard phenol/chloroform and ethanol precipitation methods [13]. Total genomic DNA (10 μg) was digested with the restriction enzymes Eco RI or Hind III, fractionated by electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane (Zeta-probe, Bio Rad, California, U.S.A.) using the Southern procedure [14].

Hybridisation was carried out under stringent conditions according to the manufacturer's description by nick translation or multiprime labelled DNA probes (Amersham, U.K.). These were labelled to a $1–2\times10^8$ cpm/μg DNA specific activity, using [α-^{32}P] dCTP. The filters were exposed to Kodak X-Omat AR-5 film for 1–7 days at −80°C using intensifying screens.

The DNA probes used were: plasmid pMC 413 RC corresponding to the c-*myc* exon III (1.4 kb Cla I-Eco RI fragment) [15]; plasmid pMAC 117 corresponding to the human gene c-*erb-B2* (1 kb Bgl II-Bam HI fragment) [16]; plasmid BK4 containing a 1.03 kb Bam HI-Kpnl fragment of the human gene *int-2* [17]; plasmid pH M2A containing a 2.7

Eco RI insert of c-*mos* [18]; plasmid pHiHi3 corresponding to the gene K-*ras* (1 kb Hinc II-Eco RI) [19].

The following oncogene-specific DNA fragments were used as probes: Ava II fragment (nucleotides 281–872) from clone pORF1 which contains most of the *hst-1* cDNA coding sequence [20]; 6 kb Bam HI fragment from the p344 clone corresponding to H-*ras* [21], 1.8 kb Eco RI-Bam HI fragment from pJB327 corresponding to the L-*myc* gene [22], 1 kb Eco RI-Bam HI fragment from pNB-1 corresponding to the N-*myc* gene [23].

Hybridisation with a probe corresponding to human *β-actin* was used as an internal standard to determine the amount of DNA in each sample.

Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis

Mutations in the genes K-*ras* (codon 12), H-*ras* (codons 12, 13 and 61), *RB* (exons 20, 21, 22) and *p53* (exons 5, 6, 7, 8) were examined using the PCR–SSCP method as described by Orita and colleagues [24]. The nucleotide sequences of the primers used are reported in Table 1. PCR (template DNA 100 ng, primers 100 pmol, dNTP 0.2 mM, DNA Taq polymerase 2.5 U) was performed in a thermal cycler (MJ Research, Massachusetts, U.S.A.) for 35 cycles at 95°C for 30 sec, at 66°C for 45 sec and at 72°C for 90 sec (*p53*), for 40 cycles at 96°C for 15 sec, at 55°C for 30 sec and at 72°C for 90 sec (K-*ras*, H-*ras* and *RB*). The final cycle was followed in each experiment by a 7 min extension at 72°C. For the SSCP analysis, the PCR products were heat-denatured and electrophoretically separated by 6% (*p53* exons 5, 7, 8; K-*ras*, H-*ras*, *RB*) or 8% (*p53* exon 6) non-denaturing polyacrylamide gel at 200 V for 2–4 h at 20°C and then visualised by ethidium bromide staining (0.5 μg/ml).

Cytofluorimetric analysis

In order to evaluate their DNA content, the cells from the SCLC-R1 cell line were prepared by resuspending a pellet of 1×10^6 viable cells in 1 ml of DAPI at 4°C for 1 min. The cells were then filtered through a disposable 40-μm filter assembly (RATCOM, Ylem, Italy). Human lymphocytes were used as an internal standard. The samples were analysed with a flow cytometer (RATCOM, Ylem, Italy). The DNA index was evaluated [25].

Chemosensitivity assay

In vitro drug sensitivity of the cells from the SCLC-R1 cell line was determined by the antiproliferative assay proposed for the first time by Tanigawa and associates [26]. Briefly, 0.5 ml of 0.5% agar in RPMI (Gibco, BRL, Life Technology, Belgium) supplemented with 10% FCS (Gibco) was added to 16×18 mm wells of a 24 well plate. The medium contained 1% L-glutamine (Gibco), 10 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). The cells (1.5×10^5/well) were suspended in 0.5% agarose in the medium, and 0.5 ml of the suspension was added to each plate. Triplicate cultures were set up for each experimental point.

The drugs [cisplatin, carboplatin, etoposide (Bristol-Myers Squibb S.p.A.), doxorubicin (Novartis), 4′-hydroperoxy-cyclophosphamide, ifosfamide (Asta Medica), docetaxel (Rhône–Poulenc Rorer), vinorelbine (Pierre Fabre Pharma)] were added to the tumour cell suspension at the appropriate concentrations immediately before plating. In each experiment, the positive control wells were plated with the addition of $HgCl_2$ to a final concentration of 1 mg/ml. After 48 h of incubation, each well was labelled with 5 μCi [methyl-^{3}H] thymidine (Amersham) and incubated for a further 24 h. The content of each well was transferred to plastic tubes, PBS was

Table 1. *Sequences of the primers for H-*ras*, K-*ras*,* p53 *and* RB *genes used for polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis*

Gene	Primer	Sequence	PCR products (bp)
H-*ras*	H-ras-12-A	5′-GACGGAATATAAGCTGGTGG-3′	63
	H-ras-12-B	5′-TGGATGGTCAGCGCACTCTT-3′	
	H-ras-61-A	5′-AGACGTGCCTGTTGGACATC-3′	73
	H-ras-61-B	5′-CGCATGTACTGGTCCCGCAT-3′	
K-*ras*	K-ras-12-A	5′-GACTGAATATAAACTTGTGG-3′	107
	K-ras-12-B	5′-CTATTGTTGGATCATATTCG-3′	
p53	p53-5-A	5′-CTCTTCCTGCAGTACTTCCCTGC-3′	211
	p53-5-B	5′-GCCCCAGCTGCTCACCATCGCTA-3′	
	p53-6-A	5′-GATTGCTCTTAGGTCTGGCCCCTC-3′	185
	p53-6-B	5′-GGCCACTGACAACCACCCTTAACC-3′	
	p53-7-A	5′-GTGTTGTCTCCTAGGTTGGCTCTG-3′	139
	p53-7-B	5′-CAAGTGGCTCCTGACCTGGAGTC-3′	
	p53-8-A	5′-ACCTGATTTCCTTACTGCCTCTGGC-3′	200
	p53-8-B	5′-GTCCTGCTTGCTTACCTCGCTTAGT-3′	
RB	RB-20-1-A	5′-TGCCTCATAATAAACCAGTA-3′	292
	RB-20-1-B	5′-TCATTCTGCAGGGTGTGCTG-3′	
	RB-20-2-A	5′-TGAGCACCCAGAATTAGAAC-3′	393
	RB-20-2-B	5′-TTAACAAGGTGTGGTGGTAC-3′	
	RB-21-1-A	5′-TAATTGAGCCTTGGTGATTT-3′	305
	RB-21-1-B	5′-CATGAGGAAGATCCTTGTAT-3′	
	RB-21-2-A	5′-TCATTGTAACAGCATACAAG-3′	333
	RB-21-2-B	5′-TCTATGACTCATGTCAAGTT-3′	
	RB-22-1-A	5′-TTTCACTTCTAGAAGAGCAG-3′	284
	RB-22-1-B	5′-CTGCATGAAGACCGAGTTAT-3′	
	RB-22-2-A	5′-CATTCAAACGTGTTTTGATC-3′	304
	RB-22-2-B	5′-GGTGAAATATAGATGTTCCC-3′	

added, placed in a water bath for 30 min at 90°C and centrifuged. The pellets were washed with PBS, treated with 10% trichloroacetic acid (TCA) for 1 h at 4°C and then dissolved in 400 µl of potassium hydroxide (0.075 N). After 2 h, the solutions were transferred to scintillation vials containing 3 ml of Pico-fluor-15 and counted (Beckman β-Counter).

The assay was considered evaluable if the average count of the untreated controls was > 300 cpm and if the positive control gave at least 80% inhibition of the thymidine uptake. The inhibition of ^{3}H-thymidine incorporation defined the cell line as markedly resistant (< 40%), moderately resistant (40–75%) and non-resistant (> 75%) to a specific drug [26].

Immunoenzymatic determination of tumour markers

The levels of carcinoembryonic antigen (CEA) and NSE tumour markers were measured in the serum of the patients and in the spent media and the cytosols obtained from the respective cell lines. The LIA-mat NSE Prolifigen (BYK-Gulden) was used to test the presence of NSE and the immunoenzymatic reaction kit (Centrocor, Boehringer Mannheim) to test the presence of CEA.

Immunohistochemical determination of tumour markers

The SCLC-R1 cells were centrifuged, fixed with acetone (10 min) and chloroform (5 min) and treated with monoclonal antibodies against chromogranin A (Nuclear Laser Medicine: SCLC-Clone 123 prediluted), the specific antigen (p105, 120, 160 kDa) common to squamous cell lung carcinoma and adenocarcinoma (Chemetron: MAb 460 prediluted), human cytokeratin 19 (Dako: BA 17, diluted 1:20) and human cytokeratin 20 (Dako: K_S20.8, diluted 1:20). Immunohistochemical staining was performed by the streptoavidin–biotin peroxidase complex method.

RESULTS

The SCLC-R1 cell line was derived from one tumour sample, grown free of mycoplasma contamination, mostly as a monolayer, and had a doubling time of approximately 62 h. By phase contrast, the cells appeared prevailingly spindle shaped and grew several layers deep with no contact inhibition. Some superficial cells tended to detach (data not shown). By electron microscopy, in sections parallel to the culture surface, the cells were flattened and spindle shaped and the cell membrane was smooth, with rare junctional complexes. The cytoplasm showed expanded rough endoplasmic reticulum cisternae and peripheral bundles of filaments and numerous lysosomal bodies. A few electron dense bodies resembling neuroendocrine granules were present in some cells. The chromatin was completely decondensed so that the nucleoli also did not show the associated heterochromatin. In perpendicular sections, single cells adherent to the substrate or group of superimposed cells were visible (Figure 1). The modal chromosome number of the SCLC-R1 cells was 46 (range 44–47). The presence of diploid DNA

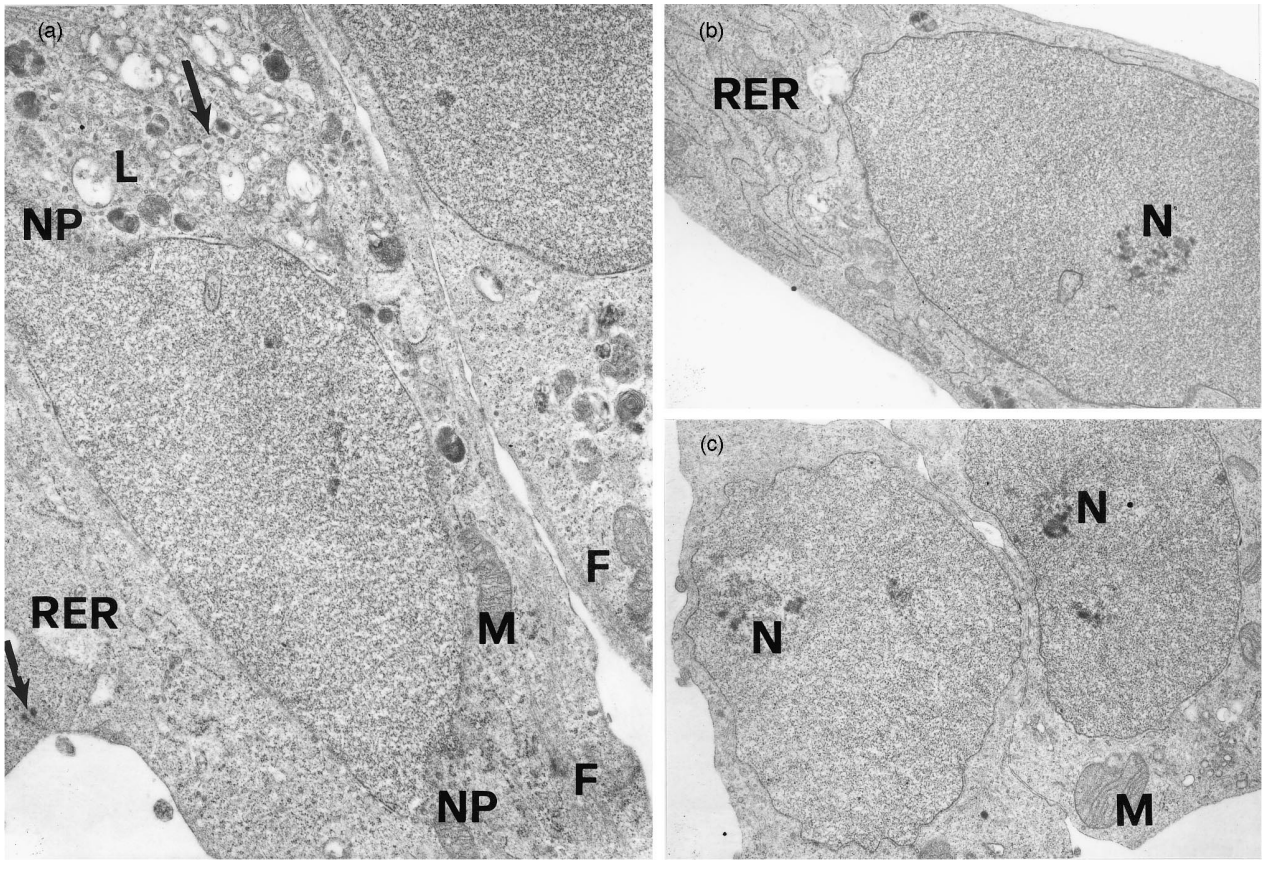

Figure 1. Ultrastructural appearance of the SCLC-R1 cell line. (a) The cells with a spindle-like shape had peripheral bundles of filaments (F) and a well developed rough endoplasmic reticulum (RER), normally shaped mitochondria (M), numerous lysosomes (L) and some electron dense granules (arrows). The nucleus, in which some nuclear pores (NP) appeared perpendicularly sectioned, shows a decondensed homogeneous chromatin, 13 000×. (b, c) Perpendicular sections showing a single cell or two superimposed cells. (b) The dilated cisternae of the RER were filled with electron dense material. The chromatin was completely decondensed, 11 000×. (c) The adjacent cells showed a decondensed chromatin and segregated nucleoli (N), 10 500×.

content was indicated by cytofluorimetric analysis. Telomeric fusions and other rearrangements occurred frequently. Always present were: a translocation involving chromosome 16 [t(16;?)(q24;?)]; the deletion of the region where the oncosuppressor *FHIT* gene has been recently localised in chromosome 3 [del(3)(p14)]; and the telomeric deletion of the short arm of chromosome 12 [del(12)(p13)] (Figure 2). The results of the FISH analysis, performed in order to validate the deletion in 12p13, indicated the presence of two signals corresponding to the probes 18G6 (95% nuclei) and 147G1 (89% nuclei) in control lymphocytes, whereas SCLC-R1 nuclei showed only the signal corresponding to the centromeric 18G6 probe (90% nuclei). Ten metaphases were also scored: all of them showed two chromosome 12 centromeric signals and only one spot on 12p (data not shown).

Genomic DNA extracted from neoplastic cells was digested with EcoRI or Hind III and hybridised by the Southern blot technique to nine different probes, homologous to cellular proto-oncogenes which are known to be involved in lung cancer: c-*myc*, L-*myc*, N-*myc*, c-*erbB-2*, H-*ras*, *int-2*, *hst-1*, c-*mos* and K-*ras*. No increase in the intensity of the hybridisation signals was evident with all the probes used. Somatic rearrangements were not present in all the sequences analysed. The presence of K-*ras* at (12)(p12) delimited at (12)(p13) the telomeric loss indicated by cytogenetic analysis (data not shown). PCR-SSCP analysis of the genes *p53* (exons 5, 6, 7, 8), *RB* (exons 20, 21, 22), K-*ras* (codon 12) and H-*ras* (codons 12, 13 and 61) did not reveal alterations and/or point mutations in the SSCP patterns of conformers.

Table 2 shows the results of the *in vitro* chemosensitivity assay performed in the SCLC-R1 lung cancer cell line for

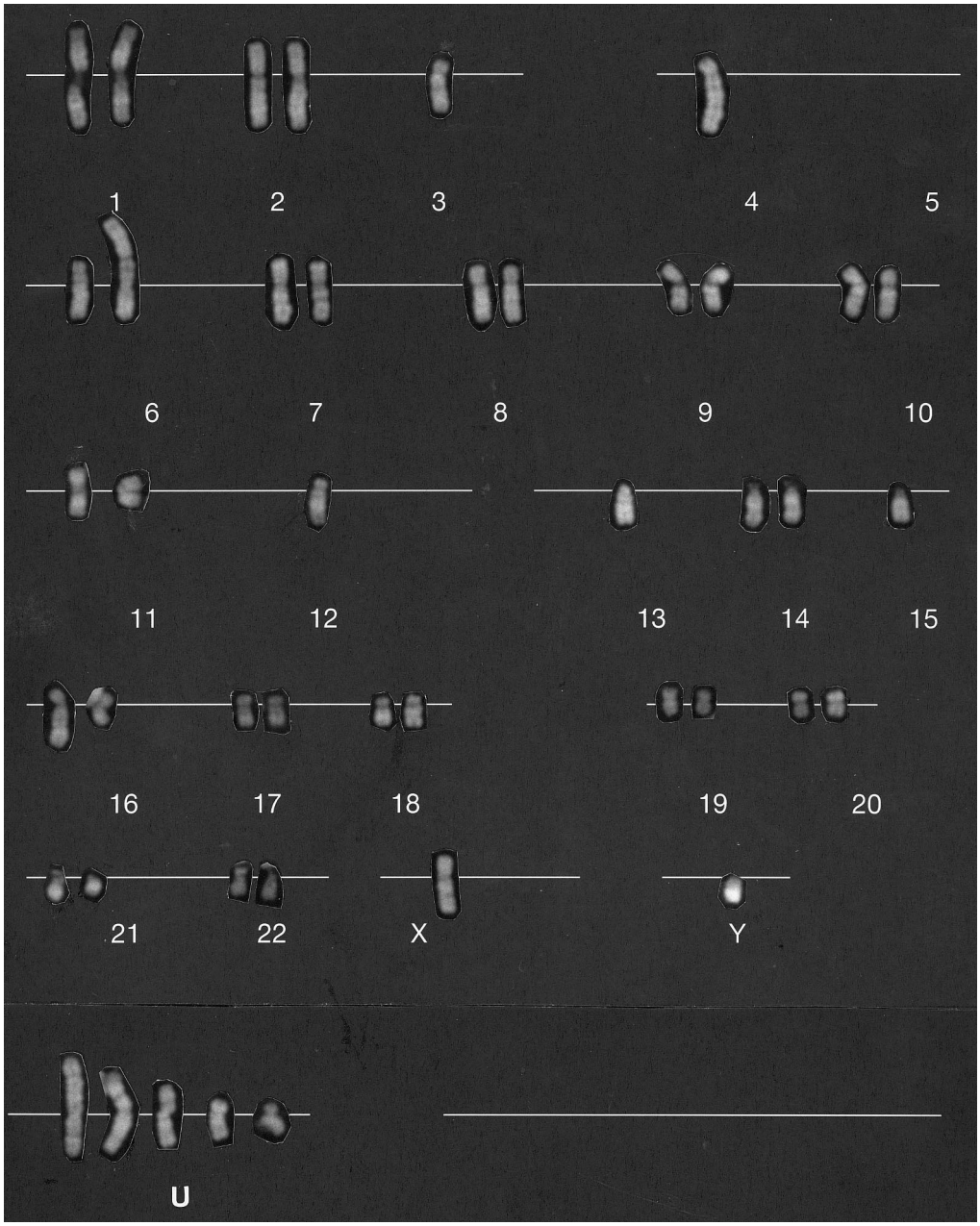

Figure 2. QFQ banding karyotype of a cell of the SCLC-R1 cell line: note the deletion of chromosome 3 [del(3)(p14)], the telomeric deletion of the short arm of chromosome 12 [del(12)(p13)] and a translocation involving chromosome 16 [t(16;?)(q24;?)].

Table 2. In vitro *chemosensitivity of the cell line*

Drugs	Doses (μg/ml)	SCLC-R1 cell line inhibition (%)
Cisplatin	2	81.2
Doxorubicin	0.4	99.1
Etoposide	1	72.4
4′-Hydroperoxycyclophosphamide	1	7.8
Docetaxel	0.1	51.5
Vinorelbine	0.05	56.0
Carboplatin	4	79.0
Ifosfamide	2	1.0

The cell line is indicated as markedly resistant (< 40%), moderately resistant (40–75%) or non-resistant (> 75%) to each drug [25].

eight drugs. The cells were most sensitive to cisplatin, carboplatin and doxorubicin.

The levels of CEA and NSE tumour markers measured by immunoenzymatic assays in the serum of the patient from which the SCLC-R1 cell line was obtained and in the cytosol and the spent media of the cell line are reported in Table 3(a). The results indicated a marked positivity of the NSE level in the cell line cytosol with respect to that present in the patient's serum, whereas the CEA tumour marker level was equally low.

The results of the immunohistochemical analyses carried out in the SCLC-R1 cell line is reported in Table 3(b). While cytokeratin 19 was highly expressed, cytokeratin 20 was not. High expression of chromogranin A, a specific marker for SCLC was detected in the cells, whereas the 105, 120 and 160 kDa human proteins, specific for NSCLC, were not.

DISCUSSION

Preventive care measures, earlier diagnosis and more effective treatments are the main research objectives to reduce both the incidence and the mortality rates of lung cancer. For these purposes and to understand better lung tumorigenesis and progression, the availability of well characterised tumour cell lines derived directly from patients with a documented clinical history and retaining properties observed *in vivo* is important. We report here the characteristics of a new cell line derived directly from one tumour sample from a patient with a metastatic lesion of a SCLC named R1. The cell line retained the morphological and histological characteristics of epithelial cells. The line's histotype was further characterised by the immunohistochemical evidence of specific antigens. The expression of the NSE in the cells reflects the neuroendocrine origin of the tumour [27].

Table 3. Tumour markers

(a) Immunoenzymatic assays

	SCLC-R1		
	Patient's serum	Cytosol	Spent medium
CEA (ng/ml)	2	1	1
NSE (ng/ml)	6.3	88.5	3.9

(b) Immunohistochemical assays

	SCLC-R1
p 105/120/160*	–
CHR-A†	+++
CK-19‡	++
CK-20	–

*Specific antigen for squamous cell and adenocarcinoma. †Chromogranin A. ‡Cytokeratin.
+++, > 90% expression; ++, 51–90% expression; –, no expression.
CEA, carcinoembryonic antigen; NSE, neuron specific enolase.

Cytogenetic analysis indicated a modal chromosome number of 46 and an extensively rearranged karyotype. Always present were noticeable deletions in chromosome 3 and 12. Deletions in chromosome 3 have been frequently reported in SCLC [4, 5], in the region where the *FHIT* suppressor gene has been recently identified (3p14.2) and exon losses in it have been found in approximately 80% of SCLC [28]. The telomeric deletion of (12)(p13) is reported here for the first time and has been validated by FISH analysis. The presence of K-*ras* in (12)(p12), as indicated by Southern blot analysis, confirmed the limit of the telomeric loss at (12)(p13). The oncogenes c-*myc*, L-*myc*, N-*myc*, *Int-2*, *erb B-2*, *hst-1*, H-*ras*, K-*ras* and c-*mos*, which have been previously found to be involved in lung cancers, were not amplified nor were there somatic rearrangements in the SCLC-R1 DNAs. In *p53*, *RB*, H-*ras*, K-*ras* genes no point mutations were evident by PCR–SSCP analysis in the SCLC-R1 cells.

The chemosensitivity tests performed in the SCLC-R1 cell line showed the cells to be sensitive to cisplatin, carboplatin and doxorubicin. This continuous cancer cell line, with its pattern of chemosensitivity, may represent a useful tool for the *in vitro* search for more effective treatments. The availability of well characterised lung tumour cell lines as fundamental models for genetic and biological studies, is important for the characterisation of molecular and cytogenetic changes in premalignant and malignant lesions and their association with specific morphological changes, with the development of drug resistance and the production of growth factors and their receptors [4].

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