Establishment and Characterization of Two New Squamous Cell Carcinoma Cell Lines Derived from Tumors of the Head and Neck

Peter G. Sacks, Steven M. Parnés, Gary E. Gallick, Zahra Mansouri, Rosemarie Lichtner, K. L. Satya-Prakash, Sen Pathak, and Donald F. Parsons

ABSTRACT

Two human cell lines were established from untreated squamous cell carcinomas of the head and neck. Line 183 was derived from a head and neck squamous cell carcinoma of the tonsil and 1483 from a head and neck squamous cell carcinoma of the retromolar trigone. Both lines grow in a cobblestone pattern demonstrating their epithelial heritage. Immunofluorescence studies and one-dimensional polyacrylamide gel electrophoresis indicated that both lines contain cytokeratins. Line 1483 is more aggressive in nude mice, has a higher efficiency for anchorage-independent growth, expresses p21\(^\text{WAF1}\) (product of the \textit{ras} oncogene) at a higher level, and is more aneuploid than 183. 1483 also grows as a multicellular tumor spheroid. Line 1483, which was established from the primary tumor of a patient with nodal metastasis, thus displays more progressed characteristics than line 183, which was established from a patient with no clinically positive nodes.

INTRODUCTION

Clinical survival rates have plateaued for patients with cancers of the head and neck, reflecting few significant advances in treatment modalities and in understanding the biology of the disease. Since the tumor represents only 5% of the total cancers in the U.S. (1), basic research has been largely neglected, although this type of tumor presents a formidable clinical challenge. We believe that tumors of the upper aerodigestive system have several features that suggest their potential usefulness as a model for HNSCC\(^2\) as well as for squamous cell carcinoma in general: (a) most human tumors are carcinomas (1, 2) and 90% of head and neck tumors also are carcinomas (2); (b) several premalignant lesions of the upper aerodigestive system are clinically recognized (3); (c) lesions of the upper aerodigestive system have several features that suggest their potential usefulness as a model for HNSCC\(^5\) as well as for squamous cell carcinoma cell lines which were derived from untreated primary tumors of the head and neck. These cells have unique biological properties which may reflect important differences in the tumors from which they were derived.

MATERIALS AND METHODS

Establishment of Cell Lines. Surgical specimens were obtained from the operating room suite and transported to the laboratory in Eagle's MEM supplemented with 10% FCS and the antibiotics gentamicin (50 µg/ml) and fungizone (5 µg/ml). Tumor tissue was rinsed in MEM containing nystatin (50 µg/ml) instead of fungizone and, under sterile conditions minced with scalps into small-sized pieces of 1–3 mm. Pieces were placed in 35- or 60-mm Petri dishes with enough medium to wet the surface and were incubated at 37°C in a 5% CO\(_2\) and water-saturated incubator. Within 3–10 days epithelial and fibroblast cells migrated from explants. Fibroblast overgrowth was controlled by cell scraping and selective trypsinization (0.05% trypsin-2 mM EDTA). During selective dissociation the dishes were monitored under a phase contrast microscope and treatment was stopped when fibroblasts but not epithelial cells were detached. When epithelial cells in the dishes reached 75–100% confluency and were free of fibroblasts, they were subcultured in conditioned medium with an initial split ratio of not more than 1:2. Line 183 was considered “established” (able to be routinely passaged) after about 4 months in culture and line 1483 after 6 months. Lines were routinely tested for mycoplasma contamination by Hoechst staining and were always negative. The characteristics of patients from whom these cell lines were established are given in Table 1. Histopathology showed that tumor 1483 was more aggressive in that it exhibited local infiltration of subjacent muscle, fascia, and glandular tissues. This tumor also had spread to one lymph node.

In Vitro Culture Techniques. Cell lines were originally subcultured in Eagle's MEM with 10% FCS and gentamicin as above. For the cell characterization in this study, the lines were maintained in DME-M12:1 with 10% FCS and gentamicin and used between passages 15 and 30. These cell lines stratify in culture and contain cell-cell junctions that make them difficult to separate into pure single cells. When single cells were needed, vigorous pipetting of dissociated cells plus a 10-min sedimentation at 1 g was performed, and the top one-half of cells was used.

Growth rate studies used single cell suspensions of log phase cells. Cells were plated in duplicate at 20 × 10\(^3\) cells/60-mm Petri dish. Cell numbers were counted on days 0, 2, 4, 6, 8, 10, 12, and 14, and doubling times were derived from the exponential growth phase. For plating efficiency on plastic, single cell suspensions were plated in 60-mm Petri dishes at concentrations of 50, 100, 500, 1000, 2500, 5000 cells (four dishes/concentration). Colonies were allowed to form for 14–21 days, fixed in 3:1 methanol/glacial acetic acid, and stained with 0.3% methylene blue. Anchorage-independent growth was assayed by suspending known numbers of log phase cells in 0.5 ml of 0.5% agarose (Seaplaque, FMC) over a 1% agarose base. Agarose was made up with DME-M12 plus 10% FCS and cell numbers varied from 50 × 10\(^3\) to 4 × 10\(^4\) cells per dish. Following gelation an additional 1 ml of medium was overlaid on the agarose, and the plates were reseed every 7–10 days. Colonies were grown for 21 days and dishes were stained with the vital experimental research has historically stressed mesenchymally derived cells owing in large part to their adaptability to \textit{in vitro} culture techniques. In this report we describe two new squamous cell carcinoma cell lines which were derived from untreated primary tumors of the head and neck. These cells have unique biological properties which may reflect important differences in the tumors from which they were derived.

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4 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MEM, minimal essential medium; FCS, fetal calf serum; DMEM, Dulbecco's minimal essential medium; DPBS, Dulbecco's phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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Table 1 Patient-tumor characteristics

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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Stage</th>
<th>Tumor histology</th>
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<tbody>
<tr>
<td>1-83</td>
<td>54</td>
<td>M</td>
<td>T3N0M0</td>
<td>Well-differentiated SCC of the tonsil</td>
</tr>
<tr>
<td>14-83</td>
<td>66</td>
<td>M</td>
<td>T2N1M0</td>
<td>Well-differentiated SCC arising from retromolar trigone</td>
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stain iodonitrotetrazolium chloride before counting (6). A colony was defined as a cluster, at least 50 μm in diameter, of red stained cells.

Multicell tumor spheroids were initiated by seeding log-phase cells (50 × 10^4 to 4 × 10^5) onto either 60-mm nonadherent Petri dishes (Lab-Tek) or onto Petri dishes (60 or 100 mm) which had been precoated with 1.25% agar in complete DMEM-F12 (7). Spheroidal aggregates formed within 1 day and were maintained either on non-adherent dishes or in suspension culture in spinner flasks.

Tumorigenicity. To determine in vivo tumorigenicity, 10^7 viable cells were injected into the lateral thoracic wall of nude mice (live mice/cell line) obtained from Harlan Sprague-Dawley. Mice were maintained in laminar flow hoods under pathogen-free conditions. When tumors were between 1 and 2 cm in diameter individual mice were killed and selected organs (described in “Results”) were processed for histology and examined for the presence of metastasis.

Chromosome Analyses. A number of modifications of standard chromosome preparation techniques were attempted to obtain ample mitoses in which optimally condensed chromosomes could be obtained. For example, mitotic shake-off 2 h after treatment with colcemid (0.04 μg/ml) yielded highly condensed chromosomes. The following protocol yielded both satisfactory mitotic index and chromosome morphology for subsequent banding. Cells were grown in L-15 medium supplemented with 10% FCS. At about 60% confluency, cultures were treated with colcemid (0.03 μg/ml) for 6 h at 37°C. Cells were dislodged with a rubber policeman, collected in 15-ml tubes and centrifuged at 1700 rpm for 5 min. The pellet was suspended in 10 ml of a 1:1 mixture of 0.075 M KCl and 1.0% sodium citrate for 20 min at room temperature. Following hypotonic treatment, the cells were centrifuged and fixed in fresh methanol/glacial acetic acid (3:1 by volume) for 10 min at room temperature and washed three times with fresh fixative. The fixed cell suspension was then processed for various banding techniques following routine procedures (8). Some slides were conventionally stained in Giemsa for chromosome counts and identification of double minutes (DM). A minimum of 50 metaphase spreads were counted for modal number and at least five G-banded karyotypes were made for each cell line.

RESULTS

Tumor tissue from patients 1-83 and 14-83 produced epithelial outgrowths that were freed of fibroblasts as discussed in “Materials and Methods.” Lines 183 and 1483 are epithelial in...
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Nature and grow in typical epithelial cobblestone patterns (Fig. 1). The lines are morphologically distinct with 1483 cells being more compact and individual cell boundaries easily recognizable. Cells of 183 are more spread and grow with recognizable cell piling occurring in nests of all sizes (Fig. 1, left). Both cell lines grow as individual epithelial nests that enlarge and coalesce in time. In the center of individual nests, phase contrast microscopy showed a piling of cells. Electron microscopy indicated that the piling is epithelial stratification with cells being elongated and joined by desmosomes. The qualitative impression was that 183 cells have more desmosomal junctions than 1483 cells, but normal squamous differentiation was not found (Fig. 2). The uppermost layer of cells are not terminally differentiated, i.e., squames are not formed. The major ultrastructural differences found between lines 1483 and 183 were that 1483 cells contain more mitochondria and their nuclei have more prominent heterochromatin. In addition, 183 cells spread more as is evident from the thickness of equivalent cell layers (Fig. 2). Necrotic sloughed cells were found in 1483 cultures; at high magnification these appeared to have both a thickened cell membrane (cornified envelope) and an abundance of filamentous material (data not shown). This suggested that differentiation, although it is atypical, occurs to a limited extent.

Cell Growth. Growth related parameters are shown in Table 2. Cell line 1483 plated on plastic and grew in 0.5% agarose with a higher efficiency than line 183. Both lines had similar growth rates. Although clonal growth can be obtained, lower split ratios of 1:3 to 1:5 maintained healthier appearing cells for routine passaging, probably because of their epithelial nature and nest-like growth pattern with intercellular cooperative effects.

Chromosome Analysis. As shown in Fig. 3, cell line 183 is close to triploid (range, 60–80) with a bimodal distribution at 69 and 73 chromosomes. Cell line 1483 is close to tetraploid (range, 60–100) with a modal chromosome number of 74. A variety of harvesting techniques were attempted to obtain metaphase spreads suitable for banding since the standard procedure produced supercondensed chromosome morphology.

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Table 2

<table>
<thead>
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<th>Colony formation (%)</th>
<th>Tumorigenicity* (nude mice)</th>
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<tr>
<td>Doubling time</td>
<td>Plastic (X, range)</td>
<td>Agarose (X, range)</td>
</tr>
<tr>
<td>183</td>
<td>~36 h</td>
<td>~3% (0.5–7.0)</td>
</tr>
<tr>
<td>1483</td>
<td>~36 h</td>
<td>~6% (1.6–8.4)</td>
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* Growing tumors formed within 4 weeks.

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Fig. 2. Electron micrographic overviews of confluent monolayers of lines 183 and 1483. Three layers of stratification are evident in (top) line 183 (4400 x; bar, 5 μm); inset, higher magnification at arrow (41,400 x; bar, 240 nm) and (bottom) line 1483 (4400 x; bar, 5 μm).
Giemsa-banded karyotypes of cell lines 183 and 1483 are shown in Fig. 4. The two cell lines share three marker chromosomes: M1 = del 1(p21), M2 = del 6(q22), and M3 = (13q;13). Each cell line has several unique markers. Cell line 183 has four such markers: del(7q), del(11p), t(2p;?), and t(20p;?) (Fig. 4, top arrows). Cell line 1483 has six unique marker chromosomes: del(3p), t(5p;?), t(10p;6p), t(15p;17q), t(18q;?), and t(14p;ABR) (Fig. 4, bottom). These anomalies were clonal for both cell lines. Other alterations were nonclonal and could not be identified in some cases. Giemsa-banding patterns confirmed that both cell lines were of human origin.

Keratin Analyses. Both cell lines were examined for the presence of cytokeratins by indirect immunofluorescence techniques using an anticytokeratin monoclonal antibody, PKK1. As shown in Fig. 5, both lines contain an extensive cytokeratin network that reflects their epithelial heritage. The keratin content was further analyzed by one-dimensional SDS-PAGE. Since keratin polypeptide content is known to vary with differentiation state (12, 13), confluent monolayers were used for the keratin extractions for more precise comparison of the two cell lines. An example of an SDS electrophoretic gel in which 20 µg of protein/lane were analyzed is shown in Fig. 6. Immunoblotting with the broad spectrum anticytokeratin monoclonal antibodies AE1-AE3 confirmed that these bands in the M, 40,000 to 70,000 range were keratins (data not shown).

Tumorigenicity. To determine whether these cell lines were tumorigenic, 10⁵ viable cells were injected into nude mice (five mice/cell line). As is shown in Table 2, both lines produced tumors. Histologically, line 183 produced more highly keratinized tumors (Fig. 7, top) with many areas of pearl formation (pathological diagnosis of well differentiated SCC). Tumors from animals injected with line 1483 were histologically more diverse with the majority of the tumor being more undifferentiated (Fig. 7, bottom) but individual cell keratinization and areas of overt keratinization could also be found (pathological diagnosis of moderately differentiated SCC). Line 1483 displayed a higher malignant potential in that two mice developed rapidly growing tumors and died within 7 weeks. We used the anterior lateral thoracic wall as the site of tumor inoculation since this site was reported to enhance spontaneous metastasis (14). Mice were maintained up to 7 months and then killed. Autopsies were performed and selected organs (lung, liver, axillary lymph nodes, kidney, ovary, spleen) were examined histologically for metastases. In one mouse injected with cells of line 1483, a metastatic deposit was found in a vessel adjacent to a lymph node.

Spheroid Formation. Cell lines were tested for their ability to aggregate, a property that has been correlated with the transformed state (15). When plated on nonadherent surfaces, both lines aggregate and form tightly compact spheroidal masses. These were similar in morphology to aggregates produced by such tumor spheroid-forming lines as V-79 and EMT6. We attempted therefore to culture these aggregates both in stationary and suspension cultures to test whether they had spheroid forming capacities. Line 183 aggregates could not be propagated as spheroidal masses and died after several days. Line 1483 aggregates remained viable on either nonadherent surfaces or in spinner flasks and grew as multicellular tumor spheroids.

p21⁰ Determinations. Since the elevation of p21⁰ gene products in some types of tumors may correlate with the stage of malignancy, we determined levels of p21, the protein products of the c-ras oncogene, as described in "Materials and Methods." The antibody Y13-259 (16) recognizes all p21⁰ proteins of the c-ras genes. In Fig. 8, p21⁰ levels of lines 183 and 1483 were compared to LS180, a colon carcinoma elevated in p21⁰. Line 1483 showed an increased p21⁰ level compared with that of line 183. Densitometric scanning showed the increase to be approximately 25-fold.

DISCUSSION

Historically, some of the oldest tumor cell lines are from the head and neck. In the 1950s, KB from the floor of the mouth (17) and Hep 2 and 3 (18) from larynx and neck nodes (buccal mucosa primary), respectively, were developed. Now however, these lines are considered to be Hela contaminated and this, coupled to their 30 years in culture raises questions about their suitability as models for studying tumors of the head and neck. Interest and success in developing carcinoma cell lines has recently been renewed. In 1978, Krause and colleagues at Ann Arbor, MI, presented their initial work of establishing HNSCC cell lines using explant outgrowth techniques with primary or xenografted primary tumors. They produced three cell lines from 194 patients with short term epithelial culture of an additional nine patients still in progress (19). They continued this work and now have perhaps the largest collection of HNSCC cell lines (20). The establishment of carcinoma cell lines is considered difficult and low success rates have been reported. For example, three esophageal carcinoma lines were established by one group using 100 specimens (21), and recently another group reported the establishment of two head and neck lines from more than 100 specimens (22). In 1981 two teams reported the development of HNSCC cell lines. With explant
Fig. 4. G-banded karyotypes. Unique chromosomes (arrows) and three shared markers M1, M2, M3 are described in text. Top, line 183; bottom, line 1483.
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Fig. 5. Immunofluorescent micrographs of lines 183 (left) and 1483 (right) stained for cytokeratins. (1100 X; bar, 10 μm).

Fig. 6. One-dimensional SDS-PAGE analysis of cytokeratin content. Lane 1, (standards) phosphorylase B, M, 92,500; bovine serum albumin, M, 66,200; ovalbumin, M, 45,000; carbonic anhydrase, M, 31,000; soybean trypsin inhibitor, M, 21,500; lysozyme, M, 14,400. Lane 2, line 1483. Lane 3, line 183.

Outgrowth techniques similar to the methods we have used, a series of cell lines designated HN1-HN10 were derived from patients who had received radiotherapy, with or without chemotherapy (23). The second group used feeder layer techniques to establish two carcinoma cell lines of the epidermis and four of the tongue (24). One patient with tongue carcinoma (SCC-4) had received prior radiation and chemotherapy and three had not been treated (SCC-9, -15, and -25). These lines, developed by using inactivated 3T3 mouse cell feeder layers, displayed a fibroblast growth dependence ranging from very strong dependence (SCC-9) to total independence (SCC-25), and one of the epidermal carcinoma lines exhibited total dependence. Another research group recently established four new HNSCC cell lines using outgrowth techniques on tumors from patients who had received radiotherapy (25). Thus, while the number of available HNSCC cell lines is expanding, there are still a limited number in existence and fewer which are useful as models for development and progression of this important type of tumor.

Tumors are well known to be heterogeneous in a number of parameters (26). Radiotherapy and chemotherapy, by eliminating sensitive cell populations, may alter a tumor's in vivo progression and cell heterogeneity (27). The two new HNSCC cell lines we described here were established from untreated primary tumors and we believe that such cell lines are more likely to represent the initial tumor than lines derived from therapeutically treated patients. When nine HNSCC cell lines were karyotyped, those from untreated patients had lower modal numbers of chromosomes than those derived from treated patients (28). Karyotypic analyses of our cell lines demonstrated that they are bona fide lines and are neither cross-contaminated with each other nor contaminated with Hela.

As was shown in Table 1, line 1483 was developed from a primary tumor that was more advanced and histopathologically more aggressive than the tumor of line 183. This behavior was maintained by the 1483 cells in culture. The cell line was more aggressive in nude mice in that two animals developed rapidly growing tumors and died within 7 weeks; a 1483 spontaneous metastasis was found. In addition, line 1483 plated more efficiently on plastic and in soft agarose. Chromosome analyses showed 1483 to be more aneuploid and to contain more marker chromosomes. The level of p21, product of the ras oncogene, was higher and we have also reported that the level of epidermal growth factor receptor is also increased in 1483 as compared to 183 (29). Lastly, line 1483 grows as a multicell tumor spheroid and 183 does not.

Multicell tumor spheroids are believed to be of an interme-

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Fig. 7. Histological sections of nude mouse tumors. Top, representative section of 183 tumor showing several areas of terminal differentiation (pearl formation); bottom, section of 1483 tumor showing major histological pattern of undifferentiated HNSCC. As discussed in “Results,” areas of terminal differentiation can be found (not shown) (220 ×; bar, 50 μm).

mediate complexity to monolayer cultures and in vivo tumors (30). Although both normal and transformed human cells may aggregate, only certain tumor cells will grow as spheroids. In a study of 20 surgical specimens 16 formed spheroids (31), when 22 xenografts were tested only five were capable of spheroid growth (32) and in another test of 27 lines, only 16 formed spheroids (33). Normal cells may aggregate but they do not normally grow with one exception being cells of lymphoid origin (33). Of our two lines, both aggregate but only 1483 will grow. To our knowledge, this is the first report of human squamous cell carcinoma cells growing as tumor spheroids. Normal keratinocytes have an anchorage dependence for growth and the possibility that squamous cell carcinomas also have this need may account for poor growth of cell lines and in vivo tumors in semisolid medium (34, 35). The ability of line 1483 to grow as a tumor spheroid is probably mechanistically related to its increased ability to grow in anchorage-independent conditions. Another recently developed HNSCC cell line also shows spheroid forming capacity and this line also grows in anchorage independence with an efficiency of about 5%. In addition, both lines 1483 and 183 grow in anchorage-independent conditions at higher rates than that reported for other HNSCC cell lines (24, 25). In anchorage-dependent studies, however, colony formation was significantly lower than that reported by one group (25) but probably equivalent to a series of tongue carcinomas in which three out of four cell lines were highly dependent on fibroblast conditioning for clonal growth (24).

Immunofluorescence and one-dimensional SDS-PAGE analysis demonstrated that both cell lines contained cytokeratins. Keratin analysis did not detect high molecular weight keratins (M, 65,000–67,000) which are characteristic of terminal squamous differentiation (12, 13). This agrees with the morphological results from electron microscopic analysis (Fig. 2) in which normal squamous differentiation was not found to occur in cell culture. In line 183 some superbasal cells contain increased amounts of tonofilaments as shown in Fig. 2B, but

* Unpublished observations.
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Fig. 8. Immunoblot of immunoprecipitated p21. Lane 1, LS180, a colon carcinoma elevated in p21; Lane 2, line 183; Lane 3, line 1483. LC, light chain IgG.

most superficial cells were similar in appearance to the more undifferentiated cells of Fig. 2B. Whether cells with increased tonofilaments are indicative of partial keratinization or solely represent tumor heterogeneity is at present unknown.

Using a broad spectrum antibody which recognizes all c-ras proteins (16), cell line 1483 was found to have approximately a 25-fold increase in p21. It is recognized that both overexpression of the normal c-ras protooncogene or activation of the protooncogene may lead to tumorigenesis (36). The biological significance of the increase in p21 in line 1483 is unknown, and ras levels have not been looked for in other HNSCC cell lines. In one of the few studies in which head and neck cancers were examined for oncogenes, increased transcriptional levels of Ha-ras and Ki-ras were found (37). The ras oncogenes have been correlated with such biological functions as anchorage independence and growth factor secretion; line 1483, which has the higher level, is biologically more progressed. In addition, we have found that epidermal growth factor receptor, analogous to the c-erb-B oncogene, is also elevated in line 1483 (29), similar to the elevation of epidermal growth factor receptor reported in some HNSCC cell lines (38, 39). This overexpression was related to increased RNA whereas DNA from these lines showed neither gene amplification nor rearrangement (40).

In Giemsa-band analyses we found several unique markers for each line and three shared markers. Although as discussed above, several groups have established HNSCC cell lines, detailed chromosomal analyses have not been reported for most. A study of six of the HN series (28), showed abnormalities on chromosome 1 to be common to all. Our two cell lines also have a deletion in chromosome 1 and whether aberrations on chromosome 1 will be important for the development of HNSCCs is at present unknown. Aberrations on chromosome 1 have been correlated with hematological malignancies (41), and several oncogenes, Nras (42), the human homologue of chicken v-SK (43), B-Lym (44), and cpx (45) have been mapped to chromosome 1.

The two HNSCC cell lines described in this report differ in a number of biological and biochemical parameters. We view cell lines as tools with which studies aimed at understanding squamous cell carcinoma biology can be undertaken using a variety of biochemical and biological systems, as has been done with the HN series of cell lines (46). These two cell lines (183 and 1483) show good tumorigenic potential in nude mice, and this, coupled to their increased ability to grow under anchorage-independent conditions, suggest their potential usefulness in therapeutic based studies. The ability of one of these lines to grow as a multicellular tumor spheroid also opens this model and its numerous applications (47) to studies aimed at a better understanding of squamous cell carcinomas of the head and neck.

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REFERENCES


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