Characterization of the tumor cells from human head and neck cancer

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Abstract
Although it is very difficult to establish a correlation between the behavior of tumor cells in vitro and in vivo tumors, in this study we tried to analyze some functions of the tumor cells isolated from head and neck tumor fragments, comparatively with the tumor cell cultures, hoping that the results could help the clinician to choose optimal treatment for head and neck cancer patients. The aim of the present study was to evaluate the expression of p53 protein, the apoptosis process and the cell cycle parameters in the cell cultures of head and neck carcinoma obtained from fragments of tumor excised from patients. Using ELISA assay and flow cytometry methods we analyzed the p53 protein expression, distribution of the cell cycle phases and the level of apoptosis in the tumor cells which were immediately isolated from the tumor fragments, as well as in the ones that were subsequently passed in culture several times. Our results showed that p53 protein expression was weak in four (33.3%) patients, moderate in three (25%) patients and intense in five (41.6%) patients. Our data showed that both tumor cells isolated from the tumor fragments (passage 0), as well as cell cultures at passage 5 had the percentage of S-phase of the cellular cycle was weak in four (33.3%) patients, moderate in three (25%) patients and intense in five (41.6%) patients. Our data showed that both tumor cells isolated from the tumor fragments (passage 0), as well as cell cultures at passage 5 had the percentage of S-phase of the cellular cycle.

Keywords: squamous cell carcinoma, p53, apoptosis, cell cycle, flow cytometry.

Introduction
Head and neck squamous cell carcinoma (HNSCC) accounts for 5% of all adult cancers worldwide and is the most common head and neck neoplasm in adults. It constitutes nearly 2–3% of all malignant tumors after lung cancer [1]. Survival rates for patients with advanced head and neck cancer have remained almost unchanged in the last years. This situation determines the availability of new prognostic factors that could guide the treatment modalities in the context of clinical studies and facilitate the extent of the initial operative procedure. Additional reliable prognostic indicators that may be useful in selecting subgroups of patients who receive specific therapy should be considered [2, 3]. The possibility to characterize the tumor cells isolated from tumor fragments of head and neck cancer patients compared to characterization of tumor cell cultures may help the clinician to choose the optimal treatment for a specific tumor. Tumor tissues can be dissociated into their component cells, from which individual cell types can be purified and used for biochemical analysis or for the establishment of cell cultures. Many animal and plant cells survive and proliferate in a culture dish if they are provided with a suitable medium containing nutrients and specific growth factors [4–6].

P53 is a tumor suppressor gene, located in 17p13, which encodes a nuclear protein. The P53 gene has an important function in the regulation of the cell cycle, DNA repair and programmed cell death pathways [7, 8]. Carcinogenesis appears to be a consequence of a combination of disturbances in signal transduction pathways that control cell cycle checkpoints, cell survival, arrest and apoptosis [9–12]. The program for the cell growth and cell division (proliferation) called “cell cycle” consists of a series of events that are involved in the growth, replication and division of cells. Cell cycle can be subdivided into two major stages, interphase and mitosis. Interphase is characterized by three successive phases G1, S and G2. During G1 (Gap 1) phase, the RNA and protein synthesis occur. In this phase, cells are able to grow and to produce all the necessary proteins for DNA synthesis. Once the S (Synthesis) phase has started, DNA begins being synthesized. The G2 phase (Gap2) follows in cells which continue to grow and to prepare for mitosis (M phase), the final phase of the cell cycle. During M phase, the cell splits through cytokinesis into two daughter cells. Following mitosis the cells may re-enter the G1 phase or proceed to “G0” phase, where growth and replication stop. Malfunctions in cell division pathways can lead to increased cell division, tumor formation and carcinogenesis [13, 14]. Cancer cells trick the cell division control system and information about the cell cycle parameters (G1, S, G2) are crucial for understanding the causes of increased cellular division and development of
cancer. Cellular proliferation depends on the rates of both cell division and cell death. Genetic changes lead to the loss of programmed cell death (apoptosis) and are likely to be critical components of tumorigenesis. Apoptosis is a mechanism which condemns cells to death in order to control cell proliferation or as a response to DNA damage. It has been suggested by some researchers that cells with a diminished apoptotic response have an increased potential to transform normal cells into the malignant phenotype. Therefore, the aim of the present study was to characterize the primary cell cultures obtained from head and neck tumors by analyzing the expression of P53, the cell cycle parameters and apoptosis processes in order to obtain information about prognosis or sensitivity to therapy.

Materials and Methods

Reagents and kits

The following reagents and kits were used: human total P53 ELISA kit (R&D Systems, Minneapolis, USA), Aprotinin, Leupeptin, Pepstatin, Glycerophosphate, Sodium Azide (NaAz), Sodium Fluoride (NaF), Triton X-100 (all purchase from Sigma-Aldrich, USA); Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA), 96-well microplates, plate sealers (R&D Systems Minneapolis, USA); Annexin V-FITC kit (BD Pharmingen, USA); phosphate-buffered saline (PBS) pH 7.2–7.4; RPMI-1640 Medium – with Folic Acid supplemented, Penicillin and Streptomycin, L-Glutamine, fetal bovine serum (all purchase from Sigma-Aldrich, USA); PBS/2 mM Ethylenediaminetetraacetic Acid (EDTA) solution; wash buffer – 0.05% Tween 20 in PBS, pH 7.2–7.4 (R&D Systems, Minneapolis, USA); block buffer – 1% bovine serum albumin (BSA), 0.05% NaAz, in PBS, pH 7.2–7.4; lysis buffer – 1 mM EDTA, 0.5% Triton X-100, 10 mM NaF, 150 mM NaCl, 20 mM Glycerophosphate, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 3 μg/mL Aprotinin in PBS, pH 7.2–7.4; substrate solution – 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (3,3′,5,5′-Tetramethylbenzidine – TMB) (R&D Systems, Minneapolis, USA); stop solution – 2 N H2SO4 (R&D Systems, Minneapolis, USA); Propidium Iodide (PI) stock solution: 4 mg/mL; PI in PBS; RNase A stock solution: 10 mg/mL (Sigma-Aldrich, USA).

Isolation and culture of tumor cells from tumors

We used a method for generating cell cultures of human carcinoma cells from tumoral fresh tissues, which were obtained from patients with head and neck cancer after resection of tumors. The biological samples were obtained from patients with larynx or pharynx cancer (squamous carcinoma) who underwent surgery during the years 2013–2015 at “Prof. Dr. Dorin Hociotă” Institute of Phonaudiology and Functional Surgery, Bucharest, Romania. Summaries of the pathology reports for the individual tissue specimens used in these studies are as follows: specimens from patients (P) noted with P2, P8, P10 were characterized as well-differentiated, infiltrating, squamous cell carcinoma from the primary larynx cancer specimen; P4, P12 – well-differentiated, infiltrating, squamous cell carcinoma from the primary pharynx cancer specimen; P5, P7, P9 were characterized as moderately-differentiated squamous cell carcinoma from the primary larynx tumor. P1, P3, P11 were characterized as poorly differentiated squamous cell carcinoma from the primary larynx tumor and P6 was characterized as poorly differentiated squamous cell carcinoma from the primary pharynx cancer specimen. Fresh tissue was transferred into sterile transport-medium complemented with antibiotics and 0.25 μg/mL Amphotericin B as antifungal. The samples were rinsed with sterile culture medium (RPMI 1640, Sigma-Aldrich, USA) supplemented with antibiotics (100 U/mL Penicillin and 100 μg/mL Streptomycin), L-Glutamine 2 mM, and 10% fetal bovine serum, cut with scissors and dissociated into small explants which were seeded into dishes providing a surface suitable for cell attachment. The attached explants were incubated at 37°C with a humidified atmosphere of 95% air and 5% CO2. In order to separate the tumor cells from mixed cells suspension, our approach was based on monitoring the tendency of tumor cells to adhere strongly to the surface of the flask. When 30% cell confluence was reached, tissue particles were transplanted. When adherent cells become semi-confluent, subculture is detached using 2 mM EDTA/Tryptsin solution (Sigma-Aldrich, USA). The cells were cleaned with Hank’s solution and immediately transferred back into the culture medium containing 10% fetal bovine serum.

Cell viability

For counting viable cells, Trypan blue was used. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells incorporate the dye. The cell suspension is diluted and 20 μL of cells are mixed with 20 μL of Trypan blue suspension (0.4%). A small amount of Trypan blue-cell suspension is transferred to both chambers of the hemocytometer. Starting with first chamber of the hemocytometer, all the cells found in the 1 mm² center and in the four 1 mm² corners, keeping a separate count of viable and non-viable cells. Counting four corner-squares and middle-square in both chambers and calculate the average. Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm³ or 10⁻⁴ cm³. Since 1 cm³ is equivalent to approximately 1 mL, the total number of cells per mL will be determined using the following calculations:

Cells/mL = average cell count per square × dilution factor × 10⁴

Total cells = cells/mL × the original volume of fluid from which the cell sample was removed

% Cell viability = total viable cells (unstained)/total cells × 100

Elisa assay

ELISA kit contains the basic components required for the development of sandwich ELISA to measure human p53 protein in cell lysates (R&D Systems, Minneapolis, USA). An immobilized capture antibody specific for human p53 binds to protein. After washing away unbound material, a biotinylated detection antibody specific for human total p53 is used to detect total p53 proteins, utilizing a standard Streptavidin–HRP (Horse-radish peroxidase) system.
Flow cytometry assay

Flow cytometry is an widely assay used for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in heterogeneous populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. This technique is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies or ligands that bind to specific cell-associated molecules. Cell monolayers were removed from the flask, washed with PBS (pH 7.2) twice, fixed in 0.3% formaldehyde in PBS one hour at 4°C, and permeabilized using 0.2% Tween 20 solution for 15 minutes at 37°C. The permeabilized cells were washed and incubated with primary antibody at working concentration for 45 minutes at 37°C. Cells were then washed and incubated with FITC-labeled affinity-purified goat anti-mouse IgG for 45 minutes at 37°C. After incubation, cells were washed twice with PBS (pH 7.2), resuspended in 1% formaldehyde in PBS, and then acquisition data were performed using a FACScan flow cytometer (Becton Dickinson, USA). The cells untreated with antibody were run in parallel and served as negative controls and we used an isotype-matched control to determine any non-specific binding of the secondary reagent to the target cells. At least 10 000 cells were gated by light scatter and collected in a list mode manner. Data analysis was performed with WinMDI software 2.9 (Becton Dickinson). The percentage of positive cells was determined on fluorescence histograms using a region defined according to cell control analysis.

Apoptosis assay

The cells undergoing apoptosis break up the phospholipids asymmetry of their plasma membrane and expose phosphatidylserine (PS), which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark of apoptotic cell death during which the cell membrane phospholipid asymmetry of their plasma membrane layer. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark of apoptotic cell death. Annexin V is a Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. By conjugating FITC to Annexin V, it is possible to identify and quantitative apoptotic cells by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence/FL1) and the non-vital dye Propidium Iodide (red fluorescence/FL2) allows (bivariate analysis) the discrimination of viable cells (FITC-PI-), early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+). The apoptosis assay was carried out with the Annexin V-FITC kit using the manufacturer’s protocol from BD Pharmingen. The assay was carried out with the Annexin V-FITC kit using the manufacturer’s protocol from BD Pharmingen. The cells suspension was washed twice with cold PBS and centrifuged at 300 g for 5 minutes. The cells were resuspended in cold Annexin V binding buffer at a concentration of 1×10^6 cells/mL. The 100 μL cells suspension (1×10^6 cells) was transferred to a 5 mL culture tube and incubated with Annexin V-FITC (5 μL) and Propidium Iodide (5 μL) in the dark at room temperature for 15 minutes. In the each tube was added 400 μL of Annexin V binding buffer and the 10000 cells/sample were collected using a FACScan flow cytometer. Data were analyzed using WinMDI software 2.9 (Becton Dickinson) and shown as two-color dot plot with FITC-Annexin V (green fluorescence, X axis) vs. PI (red fluorescence, Y axis).

Cell cycle analysis

The most commonly used dye for DNA content/cell cycle analysis is Propidium Iodide. The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for optimal DNA resolution. Cells (1×10^6 cells/mL) were washed in PBS and fixed with ethanol 70% for at least one hour at 4°C. The cells were then washed in PBS and centrifuge at 300 g, 5 minutes at 4°C. The cells were then washed in PBS and centrifuge at 300 g, 5 minutes at 4°C. The cells were incubated for 10 minutes at 37°C with 0.5 mg/sample RNase A and then was added 10 μg/sample of PI staining solution to cell pellet, mix well and incubated 10 minutes at 37°C. The samples stored at 4°C until analyzed by flow cytometry. A minimum of 15 000 events for each sample were collected using a FACScan flow cytometer and CellFIT software (Becton Dickinson) and used to determine the cell cycle phase distribution after debris exclusion.

Results

Characteristics of the tumors

Head and neck tumors samples were obtained from patients who underwent surgery during the 2013–2015 periods. Our study included 31 tumor specimens who were obtained from post-operative head and neck cancer patients who had or not received chemotherapy/radiotherapy before surgery, but only 12 of them were successfully cultured in vitro. According to the World Medical Association Declaration in Helsinki, all patients were informed about the content of this study in order to obtain consent from all subjects.

The histopathological features showing tumor size, histological differentiation and lymphatic invasion were presented in Table 1.

Table 1 – Characteristics and features of the tumor from patients with head and neck cancer

<table>
<thead>
<tr>
<th>Patients (P)</th>
<th>P1–P12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years] / Gender</td>
<td>Males: 9; Females: 3</td>
</tr>
<tr>
<td>Histological type</td>
<td>Squamous cell carcinomas (SCCs)</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>Poorly differentiated: P1, P3, P6, P11 Moderately differentiated: P5, P7, P9 Well differentiated: P2, P4, P8, P10, P12</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Larynx: 9 Pharynx: 3 None: 8</td>
</tr>
<tr>
<td>Prior therapy</td>
<td>Cisplatin + 5-FU (5-Fluourouracil): 2 Radiotherapy: 2</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>Absent nN0: 5 Present pH+: 7</td>
</tr>
</tbody>
</table>

Morphologically, all of these tumors belonged to the squamous cell type. Five of the analyzed tumors were well differentiated and showed frequent keratin pearl formation and the vesicular nuclei contained one or two nucleoli. Most tumors showed lymphocytic cell infiltration in the stroma (Figure 1).
Cultures of head and neck carcinoma cells

In vitro culture systems that allow extended growth of tumor cells are a powerful tool for the study of carcinogenesis and cancer therapy, but also for the development of new therapies and drugs. Therefore, we tried to establish a method of growing human head and neck carcinoma cells in monolayer. Maintaining cultures of human carcinoma cells was difficult, the cultures being often lost due to bacterial/fungal contamination, massive tumor cell necrosis, tumor cell senescence or fibroblast overgrowth. In our study, the cultures were obtained from 12 of 31 (39%) specimens of squamous cell carcinoma of larynx and pharynx (Table 2).

Table 2 – Characteristics and features of the tumor cell cultures obtained from fragments of human head and neck cancer tumors

<table>
<thead>
<tr>
<th>Cell cultures from tumor patients (CCP)</th>
<th>Gross type</th>
<th>Viability [%]</th>
<th>Doubling time [h]</th>
<th>No. of passages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP1</td>
<td>Adherent</td>
<td>64</td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td>CCP2</td>
<td>Adherent</td>
<td>82</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>CCP3</td>
<td>Adherent</td>
<td>71</td>
<td>96</td>
<td>3</td>
</tr>
<tr>
<td>CCP4</td>
<td>Adherent</td>
<td>87</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>CCP5</td>
<td>Adherent</td>
<td>79</td>
<td>72</td>
<td>6</td>
</tr>
<tr>
<td>CCP6</td>
<td>Adherent</td>
<td>59</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>CCP7</td>
<td>Adherent</td>
<td>73</td>
<td>96</td>
<td>5</td>
</tr>
</tbody>
</table>
Characterization of the tumor cells from human head and neck cancer

Cell tumor cultures were established from pathologically confirmed head and neck squamous cell carcinoma. In order to analyze the growth properties and morphology in vitro, population-doubling times were established and cell viability was determined using dye-exclusion Trypan blue method. For morphological studies, the tumor cell cultures were controlled daily using an inverted microscope (Figure 2). Tumor cells derived originally from culturing of the squamous carcinoma specimen presents an epithelial-like phenotype. Five of the cell cultures analyzed in sub-passages of primary cultures continued to proliferate at different growing rates (Table 2).

<table>
<thead>
<tr>
<th>Cell cultures from tumor patients (CCP)</th>
<th>Gross type</th>
<th>Viability [%]</th>
<th>Doubling time [h]</th>
<th>No. of passages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP8 Adherent</td>
<td>90</td>
<td>48</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>CCP9 Adherent</td>
<td>85</td>
<td>72</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>CCP10 Adherent</td>
<td>88</td>
<td>48</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>CCP11 Adherent</td>
<td>61</td>
<td>120</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>CCP12 Adherent</td>
<td>83</td>
<td>72</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2 – Tumor cells in culture (CCP) obtained from tumor specimen of patients (100x magnification): (A) CCP2; (B) CCP4; (C) CCP8; (D) CCP10; (E) CCP12;.
Expression of p53 protein in head and neck tumor cells

The TP53 tumor suppressor gene is one of the commonly mutated genes in pathogenesis of different types of cancers. The p53 protein is well known for its role in the control of cell proliferation, apoptosis and genetic stability. In order to investigate the p53 protein expression we used two methods, flow cytometry and ELISA assay. Our data obtained by flow cytometry show variable levels of p53 protein expression in the tumor cells of patients, which were included in this study. The distribution of positive staining was:

- IIF >30% – intensive expression;
- IIF 20–30% – moderate expression;
- IIF 10–20% – weak expression;
- IIF <10% – no expression.

Intracellular expression of p53 by indirect immunofluorescence method was analyzed in tumor cells isolated from tumor tissues of 12 patients with head and neck cancer. The intensity of p53 immunofluorescence (IIF%) of samples is shown in Figure 3A. Four (33.3%) patients showed a weak expression, three (25%) patients a moderate expression, and five (41.6%) patients an intensive expression of p53. The positivity of p53 protein was correlated with the well-differentiated histological type of the tumor; the carcinomas which are infiltrative lymph node metastasis, were p53 positive (moderate and intensive staining). Our findings suggested that the accumulation of p53 protein could serve as a significantly predictive marker of lower survival rates for patients with head and neck cancer. Flow cytometric data was confirmed by the results obtained using ELISA assay, which are presented in Figure 3B. Intracellular expression of p53 was only analyzed in five of the cell cultures established from tumor tissues of patients with head and neck cancer that had proliferated and reached passage 5. The intracellular expression of p53 data analysis in cell tumor cultures (passage 5) did not show significant differences compared to the initial moment – passage 0 (Figure 3, A and B).

Analysis of cell cycle parameters in head and neck tumor cells

An accurate description of cell cycle parameters may be important in predicting the rate of growth, prognosis and response to chemo-radiotherapy in head and neck cancer. We used a flow cytometric method for estimating the state and phase of the cell cycle in the tumor cells from carcinoma larynx or pharynx, in order to examine whether or not primary abnormalities of the cell cycle exist in head and neck neoplasia. The cell cycle analysis using flow cytometry can provide a measurement of cells actively involved in the synthesis of DNA, that is, in the S-phase of the cell cycle. The percentage of cells in the S-phase gives an indication of how fast a tumor is growing. The results of our research regarding the distribution of the cell cycle phases are presented in Figure 4, A and B.

Data analysis showed that both tumor cells isolated from the tumor fragments (passage 0) as well as cell cultures which were passed through passages 5 had the percentage of the S-phase of the cellular cycle with values between 22–35% (Figure 4, A and B). The S-phase values of tumor cells higher than 20% suggest that tumors have high proliferative activity and may be useful in determining treatment strategies.

Analysis of the apoptosis level in head and neck tumor cells

The basic cancer research has produced remarkable advances in our understanding of cancer biology and cancer genetics. Among the most important of these advances is the understanding that apoptosis and the genes that control it have a profound effect on the malignant phenotype. The oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. However, many research data indicates that other oncogenic changes promote apoptosis, thereby producing selective pressure to override apoptosis during multistage carcinogenesis. Finally, we now have enough evidence that most cytotoxic anticancer agents induce apoptosis, raising the intriguing possibility that malfunctions in apoptotic programs contribute to treatment failure. In our study, the evaluation of apoptosis level has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy. Our results showed that tumor cells cultures have a small percentage of early apoptosis (FITC+PI-/FL1) and late apoptosis or necrotic cells (FITC+PI+/FL2), which varied
from 1% to 18%. The low apoptosis level may reflect a biological aggressive tumor. In addition, we noticed that the tumors which have decreased apoptosis presented an increased cell proliferation with a high S-phase (Figure 5).

Figure 4 – (A) Cell cycle analysis of tumor cells obtained from 12 specimen tumors (P1–P12 represent number of the patient, primary culture) and five tumor cell cultures at passage 3 (B). The cells were labeled with PI and analyzed by flow cytometry. The data indicate the percentage of cells in each phase of the cell cycle (G1, S, G2/M). All experiments were performed in duplicate and gave similar results.

Figure 5 – Apoptosis level of cells from primary cultures obtained from 12 primary tumors (CCP1–CCP12 represent the cells culture of tumor patients). Cells were double stained with Annexin V-FITC and PI and analyzed by flow cytometry. The data from low right quadrant indicate the percentage of early apoptosis (Annexin V-FITC positive) and data from up right quadrant Annexin V-FITC and PI positive indicate late apoptosis. All experiments were performed in duplicate and gave similar results.

Discussion

Human head and neck cancer cell lines in cultures are used in cancer research, where experiments cannot be performed using tissue from in vivo specimens and are important tools in understanding the biology of this type of cancer. However, the establishment of head and neck squamous cell carcinoma cell lines is considered to be difficult, and low success rates have been reported [15–17]. The establishment of a head and neck cancer cell line is difficult from the primary site, on the one hand due to the possible bacterial contamination when the tumor tissue was rejected and the other hand to low cellular activity of primary tumors compared to that of the metastatic tumors. For these reasons, cell lines from primary tumors are required for understanding the biological characteristics of oral cancer [18, 19]. In our study, we successfully obtained cultures from 12 of 31 (39%) specimens of head and neck squamous cell carcinoma. The majority of specimens that did not grow were taken from tumors that are necrotic and infected. However, the cell lines described here were obtained from untreated primary tumors and our belief is that such cell lines are more likely to represent the initial tumor than lines derived from therapeutically treated patients. The cells after culturing from tumor tissue were small and round and after 3–5 days, the cells attached to the bottom of the dish, started to form branches in many directions. Two weeks later, the cultures contained fusiform, spherical or triangular cells which became 30–40% confluent (as shown in Figure 2). The in vitro experiments performed on cell carcinoma cultures in order to test the expression of selective markers could be useful for a better prognosis in head and neck cancer and in choosing the type of drugs that could be administrated to the patients [20, 21].

The transcriptional factor p53 protects normal cell growth and initiates malignant cell death. p53 can be activated by a variety of cytotoxic stresses, such as DNA damage induced by ionic irradiation and chemicals, activation or mutation of oncogenes, hypoxia and virus infection. When cells suffer toxic stresses, p53 is activated.
Defects can occur at any point along these pathways, and the mechanism of apoptosis is complex and involves many pathways. Defects can occur at any point along these pathways, leading to malignant transformation of the affected cells, tumor metastasis and resistance to anticancer drugs. Hence, apoptosis plays an important role in the treatment of cancer, as it is a popular target of many treatment strategies [32–35]. Our results have shown the aberrant decrease in apoptosis and increased cell cycle activity of tumor cells that might play an important role in pathogenesis of head and neck cancer. The apoptotic program can be manipulated to produce massive changes in cell death, therefore the genes and proteins controlling apoptosis will be potential drug targets.

### Conclusions

The results obtained after evaluating the protein expression of p53 in primary squamous carcinoma cells gathered from tumor tissue of patients with head and neck cancer sustain the hypothesis that P53 could be a predictor of prognosis and may be associated with histological differentiation grade and lymphatic invasion. A low apoptosis level in the primary culture of tumor cells was correlated with increased S-phase of cell cycle. The higher S-phase percentage in tumor cells appears to be a promising marker for predicting the evolution of head and neck cancer patients. In addition, establishing S-phase value in tumor cell cultures may be useful for diagnostic and possibly in monitoring the effectiveness of therapy. Our results suggest that both flow cytometric analysis and ELISA assay are useful tests in addition to other clinical and pathological parameters, which could help to predict the evolution of the disease and the response to therapy administrated to patients with head and neck tumors.

### Conflict of interests

The authors declare that they have no conflict of interests.

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