Cell-based diagnostic of bronchial carcinoma: case study of a diagnostic value of tests of circulating tumor cells and organoid culture

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Diagnostic based on analysis of living tumor cells is frequently used in oncology. Circulating tumor cells in the blood and cells obtained from a tumor biopsy are used to access their carcinogenic properties and subsequently to predict possible development of the disease. Here we report use of these two tests to assess aggressiveness and metastatic potential of a bronchial adenocarcinoma. The circulating tumor cells test was negative, no circulating cells was observed. It indicates that there was no metastatic spread. However, test with the surgery biopsy showed presence of aggressive cellular clones. The tumor cells from the biopsy proliferated and spread from the cultured tissue. Moreover, the tumor cells formed colonies of cells which lost contact inhibition. This is an indication of aggressive carcinogenic features of the cells in tumor organoids. Combination of both tests showed that the local tumor had an aggressive phenotype, but no detectable spreading of cells. Therefore, these tests support a management plan with removal the primary tumor and regular monitoring, without need of an extensive chemotherapy.

Keywords: Cancer, adenocarcinoma, circulating tumor cells, organ culture.

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Технології від Оранта Діагностика Раку (Oranta Cancer Diagnostics AB) було на- дано на некомерційній основі, безоплатно та як академічна наукова співпраця.

Діагностика на основі аналізу живих пухлинних клітин все частіше використовується в онкології. Циркулюючі пухлинні клітини в крові та клітини отримані з біопсії пухлин використовують для аналізу їхньої фізіології, і в подальшому для прогнозування можливого розвитку захворювання. В поданому тут клінічному спостереженні, ми повідомлюємо про використання цих двох тестів для оцінки агресивності та метастатичного потенціалу бронхіальної аденокарциноми. Тест на циркулюючі пухлинні клітини був негативним, циркулюючі клітини не спостерігалась. Це свідчить про відсутність агресивного метастатичного поширення. Однак тест з гірськіою біопсією виявив наявність агресивних клітинних клонів. Пухлинні клітини біопсії проліферували активно та мігрували з культивованих пухлинних органоідів. Пухлинні клітини утворювали колонії клітин, які втра- тили контактне інгібування. Це засвідчує наявність в біопсії агресивних ракових клітин. Поєднання обох тестів виявило, що первинна пухлина в легенях мала агресивний фенотип, але активного метастазування не виявлено. Використання цих двох тестів – інформативне для планування лікування пацієнта без потреби застосовувати агресивну хіміотерапію.

Ключові слова: рак, аденокарцинома, циркулюючі пухлинні клітини, органоїдна культура

Introduction
Tests of living cells obtained from a tumor allow evaluation of the cellular tumorigenic behavior and responsiveness to drugs (1, 2). Current standard of cancer diagnostics includes histopathological and markers tests with a tumor biopsy and body fluids, e.g. the blood, which are performed with samples containing fixed and non-living cells. Histopathological tests with sections of a tumor biopsy provide snapshots of a tumor status. Markers in the tumor or in the blood may show correlations with stages of tumorigenesis, but the confidence of prediction of development of a tumor is relatively low. To access tumor physiology and responsiveness in dynamics,
living cells are the only alternative source for testing (1-4).

Circulating tumor cells (CTCs) tests entered clinic (3, 4). CellSearch is an example of a test approved by the U.S. Food and Drug Administration for clinical use (5). Number of CTCs in the blood correlates with aggressiveness of the disease (3-5). CTCs are results of tumor cells detaching for the tumor and entering the bloodstream.

Surgery biopsy provides access to the living tumor cells in the tumor. There have been many successful trials with using tumor cells for prognostic purpose. The tumor cells are obtained by dissociation of the tumor tissues with collagenase or other proteases (7). This technique is well-developed and ensures high rate of establishing cultures of tumor cells. However, responses of tumor cells in a culture of separated cells to anti-cancer drugs may significantly differ from the outputs of application of the same drugs in treatment of a patient. That is explained by changes in cellular physiology during establishment of the cell culture. Another reason is a dissociation of the tumor cells from their matrix and lost cell-cell contacts already at the first step of culture preparation. Organ culture has been developed to preserve 3-dimensional features of a tumor, e.g. stroma and cell-cell interactions. Organ culture approach shows better correlation of cellular behavior with clinical outputs. This is explained by preservation of three-dimensional structure of cells, cell-cell contacts and presence of tumor matrix and stroma (8-10). Therefore, organ culture is a method of first choice for testing living tumor cells.

Carcinogenic properties of cells define their proliferation rate, resistance to cell death, invasiveness, ability to form a tumor and ability to metastasize (11, 12). These carcinogenic properties are crucial for diagnosis and prognosis of the disease. When living tumor cells are tested for their carcinogenic properties, diagnostic and prognostic values are high, if not even higher than traditional snapshot markers. For example, detection of aggressive cell clones is a sign of aggressiveness of the tumor. If tumor cells do not show aggressive behavior and maintain low proliferation rate and cell-cell interactions typical for non-tumorigenic cells, that may indicate that the tumor is not aggressive.

In this case report, we describe application of two tests with living cells to access metastatic potential and aggressiveness of the tumor cells.
Cluster of tumor cells were formed already after first 3 days in culture. Representative image is shown. Note single cells and clusters. Note also a large number of debris particles, formed by dead cells. B) Tumor cells started to migrate out of the organoids (non-transparent area in the image). C) Cells from clusters spread and migrate from the site of the original cluster. D) In a monolayer, cells with lost contact inhibition formed colonies of cells growing on the top of the monolayer. Two formed colonies and an area where cells started to grow on the top of the monolayer are shown. Note that the colonies are formed in areas of a confluent monolayer. Representative images are shown. Magnification is 40x.

**Materials and methods**

**Patient**

The patient is a male, 35-years old, with no previous history of cancer. The patient was treated at the Hospital No3 of Ukrainian Railways State Company (Ukrzaliznytsia). The patient consented to the tests. Management of the patient, treatment, surgery, all manipulations and monitoring were performed at the Hospital and under Hospitals license and permissions. Tests at Qatar University laboratory with cultured human cancer cells were performed under Qatar University Institutional Biohazard Committee permit QU-IBC-2019/023.

**Materials and methods**

Cell culture medium DMEM (certified for human cells culture), fetal bovine serum (toxins and virus-free, heat inactivated) and penicillin and streptomycin antibiotics were obtained from Gibco (Thermo Fisher Scientific, Sedeer, Qatar). Cell culture plastic, e.g. cell culture flasks, 24- and 96-wells plates, pipettes, were also obtained from Gibco (Thermo Fisher Scientific, Sedeer, Qatar).

**CTCs test**

PubTrans CTCs test developed at Oranta CancerDiagnostics AB was used. In brief, 8 ml
of the blood was collected in EDTA vacutainer. Cells in the blood were cultured under conditions inhibiting attachment and growth of non-carcinous cells, e.g. white blood cells, erythrocytes and normal cells shed in the blood such as mesenchymal stem cells (MSC), endothelial cells and fibroblasts. The culture medium was DMEM with 10% of fetal bovine serum, antibiotics and an additive PBT03™ to promote tumor cells growth. After 1 to 2 weeks of culturing, only tumor cells would be proliferating, while normal cells would be washed away, or not proliferating and dying. The used scale of aggressiveness is based on number of cells recovered in the test.

**Organ culture test**

Biopsy was delivered to the lab after surgery, and was split in small fragments of size less than 5 mm in any dimension. The biopsy contained significant areas of cell death/necrosis and fragmented easily into small pieces. The biopsy sample was left in a cell culture medium; DMEM with 10% of fetal bovine serum, antibiotics and an additive PBT03™ to promote tumor cells growth. Status of cells, their morphology, growth and death were monitored every second day, and documented. Analysis of cells was performed with use of inverted microscope Leica DHi-1, and images were taken with the camera Leica MC179 HD.

**Results and discussion**

Clinical data and standard diagnostic tests showed presence of a bronchial neoplasia in the upper-left lobe of size 15 x 10 mm. The patient is a male, 35-years old. Decision was made to remove surgically this neoplasia; the whole upper-left lobe was removed. Five lymphnodes were also removed. Biopsy was taken for histological examination at the Olbio laboratory (Kyiv, Ukraine). Histopathological examination showed presence of trabecular structure of the tumor. There were observed mitotic cells and nodes of the cells separated by thin layers of fibrous stroma. Invasiveness of the tumor cells in the bronchial wall was observed. Fibrotic areas with vascularization and infiltrations of macrophages were observed. At the time of the tests, no information was available about histological type, e.g. lepidic pattern or expression of mucine. CTCs test showed presence of only few cells attached to the substrate after 3 days of culture. During these 3 days, erythrocytes, platelets and most of white blood cells were washed away from the culture. Culturing was continued for next two weeks. During that time most of the white blood cells and occasional MSC, endothelial cells and fibroblasts would be identified. As these cells are normal cells, they would not proliferate. Only cells with enhanced proliferation capacity, which is typical for tumorigenic cells, would be proliferating in the culture. Figure 1 shows images of cells detected with the CTCs test in the blood of the patient (Fig. 1, A and B). An image in panel C shows an example of CTCs detection in the blood of another patient with a metastatic and aggressive cancer. Note differences in numbers and morphology of detected CTCs of this patient (Fig. 1 A and B) and of the patient with an aggressive cancer (Fig. 1C). Result of the CTCs test indicate that there was no presence of tumor cells in the blood. This result allows a conclusion that the cancer of this patient is not metastatic.

Test with cells obtained from the biopsy showed presence of highly proliferating cells. There were observed clones of cells which lost contact inhibition. These cells formed colonies on the top of cellular monolayer (Fig. 2). Clusters of tumor cells were observed already in the first 3 days (Fig.2 A). After 1 week of culture, some cells showed mesenchymal morphology and started to spread and migrate from the clusters (Fig. 2 B and C). Proliferation rate of the cells was high, with an estimate of a doubling time to be less than 24 hrs. This is comparable with the proliferation rates of human tumor cells obtained from lung (A549), breast (MCF7, MDA-MB-231, ZR75-1), liver (HepG2) and kidney (ACHN, 786-0) tumors. The fast growing cells formed a monolayer, with the most of cells confined in this monolayer. However, there were observed multiple clones of cells which lost contact inhibition and initiated growth of colony-like structures (Fig. 2 D). For 2 flasks of the cultured cells, i.e. surface area of 25 cm2, and estimated number of tumor cells between 50,000 and 100,000 cells, there were observed up to 500 clones with lost contact inhibition. We noted that as soon as cells were reaching a dense confluent, clones were detected. The cloning coefficient was estimated as 1.0% to...
5.0%. Loss of contact inhibition and formation of substrate-independent colonies in combination with high proliferation rate are typical for highly aggressive tumor cells. Cloning coefficient in the range of 10% is considered as typical for aggressive tumors (13). With the mutation rates in tumor cells estimated as $10^{-6}$ to $10^{-7}$ per base pair (14), the observed clonal frequency is in agreement with the genetic variability of the cells. Therefore, the results of the biopsy test suggest that the resected tumor contained clones of aggressive tumor cells.

The CTCs and biopsy tests contributed to assessment of the bronchial carcinoma as a non-metastatic but with clones of aggressive cells. With time, the aggressive clones could overtake the growth of the tumor and initiate invasiveness and metastases. Therefore, a combination of these two tests provided better diagnostic value that a single test would. Our results recommend to perform both tests in all cases when the blood and a biopsy may be obtained.

**Conclusion**

CTCs and biopsy tests showed diagnostic and prognostic values for the patient, in assessment of aggressiveness of the tumor cells. Notably that the disease was not aggressive and metastatic but the primary tumor contained clones of aggressive cells. This case reports supports use of both tests for prediction of development of the disease.

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