

GENETIC STUDIES TO DIAGNOSE AND ESTABLISH ADEQUATE TREATMENT STRATEGIES FOR ESOPHAGEAL CANCER

BOGDAN SANDOLACHE¹, CĂTĂLINA MARIANA LUCA², LAURA BUBURUZAN³, CĂTĂLIN GABRIEL SMARANDACHE⁴, CRISTINA VERONICA ANDREESCU⁵, ALEXANDRU SABĂU⁶, VASILE SÂRBU⁷

¹Emergency University Hospital București, ^{2,3}University of București, ^{4,5}“Carol Davila” University of Medicine and Pharmacy București, ⁶“Lucian Blaga” University of Sibiu, ⁷Clinical Hospital Constanța

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Abstract: Cancer is a complex disease which appears as a result of the progressive accumulation of genetic aberrations and epigenetic modifications which manage to escape from normal cellular control. Neoplastic cells can have numerous acquired genetic aberrations (aneuploidy, chromosomal rearrangements, amplifications, deletions, genetic recombinations and mutations leading to loss or gain of function). Aberrations lead to an abnormal behaviour common to all neoplastic cells: irregular growth, absence of contact inhibition, genomic instability and likelihood of metastasis. The genes which can undergo mutations in cancer can be divided into two main classes: genes which undergo „gain of function” mutations, known as oncogenes, and genes which present in both alleles „loss of function” mutations, known as tumour suppressor genes. Cancer may appear due to aberrations of the various genetic combinations, which can be mutant, overexpressed or eliminated. In several types of cancer, the biomarkers have improved our capacity of diagnosis, prognosis, treatment and prediction. In general, an adequate biomarker should be useful for the definition and identification of the risks during the first stages of carcinogenesis. Moreover, biomarkers can be analysed in a non-invasive, economical manner and, consequently, we should invest the search for more biomarkers. The methods used to determine the presence and staging of cancer are: immunofluorescence, Multiplex Ligation-dependent Probe Amplification (MLPA), immunohistochemistry (p53 sequencing).

Cuvinte cheie: cancer, gene, imunofluorescența, MLPA, p53

Rezumat: Cancerul este o maladie complexă care apare ca rezultat al acumulării progresive de aberații genetice și modificări epigenetice care reușesc să scape de sub controlul celular normal. Celulele neoplazice pot avea numeroase aberații genetice dobândite (aneuploidia, rearanjări cromozomiale, amplificări, deleții, recombinări genice și mutații care duc la pierderea sau câștigul unei funcții). Aberațiile conduc la o comportare anormală comună tuturor celulelor neoplazice: creștere neregulată, lipsa inhibiției de contact, instabilitate genomică și probabilitatea de metastază. Genele care pot suferi mutații în cancer pot fi împărțite în două clase principale: gene care au mutații de tip „gain of function” (mutații activatoare), cunoscute ca oncogene; și gene care prezintă în ambele alele mutații de tip „loss of function” (mutații inactivatoare), cunoscute ca gene supresoare de tumori. Cancerul poate apărea prin aberații ale diferitelor combinații de gene, care pot fi mutante, supraexprimate sau eliminate. În mai multe tipuri de cancer, biomarkerii au îmbunătățit capacitatea noastră de diagnostic, prognostic, tratament și predicție. În general, un biomarker adecvat ar trebui să fie util în definirea și identificarea riscurilor în primele stadii ale carcinogenezei. În plus, biomarkerii pot fi analizați într-un mod non-invaziv și economic și, prin urmare, este de mare valoare să investim în căutarea mai multor biomarkeri. Metodele folosite în determinarea prezenței și stadializării cancerului sunt: Imunofluorescența, Multiplex Ligation-dependent Probe Amplification (MLPA), Imunohistochimie (secvențiere p53).

Genetic Studies

Immunofluorescence is a biological test which combines the use of antibodies and fluorescent molecules in order to identify specific objectives in the cells and tissues. The technique is very sensitive and versatile and has many applications in the fields of immunology, cellular morphology, genetic diagnosis and histopathology.

Information molecular markers: oncogenes and tumor suppressor genes

In cancer, oncogenes and tumour suppressor genes can present a specific association with the type of tumour. These genes can be altered during carcinogenesis by different types of mutations such as point mutations, chromosomal translocations, genetic amplification or gene deletion. Moreover, these genes can be analysed on different levels – DNA, RNA or proteins.(1)

Oncogenes are therapeutic targets in translational medicine. The oncogenic proteins in the cancer cells can be the target of some small molecules and when they are expressed on

¹Corresponding author: Bogdan Sandolache, Str. Splaiul Independenței, Nr. 169, Sector 6 București, România, E-mail: bogdans67@yahoo.com, Tel: +40722 666308

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the cellular surface, they can be the target of some monoclonal antibodies.

Based on the study of the data in the specialty literature which enables the correlation of the histological and immunohistochemical results, the following molecular markers have been selected for the immunofluorescence tests: i) *EGFR* și *HER2/neu* as oncogenes; ii) *p53* and *APC* as tumour suppressor genes; *p53* is a gene which codes for a protein involved in the control process of the cell cycle and it has been very useful for the identification of the genetic changes at the level of the cell cycle.

Highlighting the molecular changes at the level of the activation of the oncogenes (immunohistochemical studies)

The immunohistochemical analysis of the tumoral esophageal tissue shows a neoplastic infiltration of epithelial cells in the connective tissue in the case of patient P1. The hematoxylin and eosin staining is histopathologically characterized by a neoplastic infiltration in the perivisceral cellular adipose tissue at the level of the terminal esophagus.

Most oncogenes code proteins which are part of the signaling pathways. They can be classified into two major groups: non-receptor protein kinase and guanosin triphosphate binding proteins.(2,3)

Two molecular markers (oncogenes) have been designated to perform this activity: HER2 and EGFR, which were examined using immunofluorescence in samples of esophageal tumoral tissue and normal tissue.

HER2/neu (also known as **ErbB-2**) is the coding gene for "Human Epidermal Growth Factor Receptor 2". It is a member of the ErbB protein family, being a tyrosine kinase involved in signaling pathways for cell growth and differentiation. It is a proto-oncogene located at the long arm of human chromosome 17 (17q21-q22).

The overexpression of HER2 has been reported in only 7.7% of the esophageal carcinomata and in only 1 out of the 6 cases of esophageal adenocarcinomata.(4) The activation of Neu increases tumor cell motility, protease secretion and invasion, and modulates the checkpoint function of the cell cycle.

An overexpression of HER2 (figure no. 1) can be seen in the cases of esophageal squamous cell cancer under study, in comparison with the normal tissue samples taken from the same patient (figure no. 2). Figures no. 3 and 4 present immunofluorescence images on esophageal tissue taken from patient 5. HER2 has a continuous perimembranous disposition

Figure no. 1. Immunofluorescence for HER2/neu in normal tissue

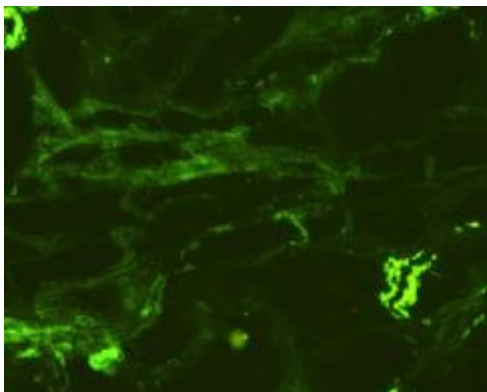
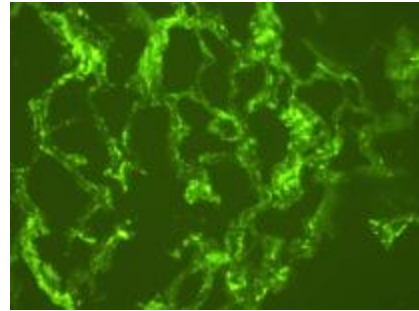


Figure no. 2. Immunofluorescence for HER 2/neu in esophageal cancer



The immunofluorescence method makes it possible to obtain information which enables the assessment of the continuity of the staining along the membranes of the tumour cells and of the number of immunostained cells which will be very useful for the oncologist during the anti-EGFR treatment.

EGFR (Epidermal Growth Factor Receptor), also called ErbB-1, HER1 in humans, is a cell-surface receptor, a member of the receptors of the epidermal growth factor family (EGF-family) which have extracellular protein ligands.(5) It is closely correlated with the receptor tyrosin kinases: HER2/c-neu (ERB2), HER3 (ERB3) and HER4 (ERB4). In cancer, the EGFR gene presents mutations which affect both its expression and its activity.

The continuous perimembranous disposition of EGFR protein and the positive reaction of the esophageal tumoral tissue to the treatment with EGFR antibodies can also be seen in the case of the patients included in this study (figure no. 3). As far as the normal esophageal tissue is concerned, the immunofluorescence reaction was negative (figure no. 4).

Figure no. 3. Immunofluorescence for EGFR in normal esophageal tissue

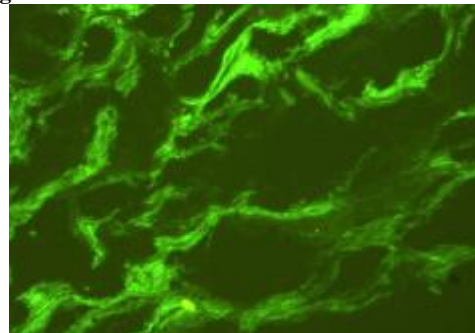
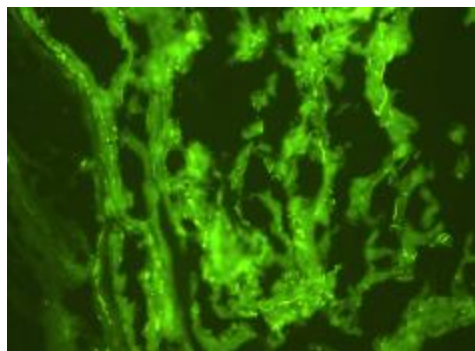


Figure no. 4. Immunofluorescence for EGFR in esophageal cancer



Identification of the possible genetic mutations at the level of the control process of the cell cycle (immunohistochemical studies)

The most frequently noticed genetic changes in human tumours affect a nuclear phosphoprotein with 393 aminoacids, known as protein 53 according to its molecular mass. Mutations of the p53 gene have been noticed in more than 50% of the human tumours. Defects of the p53 gene on the germination line lead to a tendency to develop various tumours, especially tumours of the connective tissue.

There is a strong correlation between tumour formation and the mutations of p53 gene which indicate the fact that protein 53 has a central function in tumour pathogenesis.(7,8) As a result of the research concerning the structures and functions of the coded gene and proteins, protein 53 is known as a major component of the regulating network in which control of the cell cycle, DNA integrity and programmed cell death (apoptosis) play a central part.

The mutations at the level of p53 gene are among the best known genetic lesions described in cancer. P53 functions in a homotetrameric complex as a transcription factor which induces gene expression and can facilitate the arrest of the cell cycle, DNA repair and apoptosis.

The increase of intracellular concentration of p53 can be shown through immunohistochemistry. Numerous immunohistochemistry studies have revealed that more than 50% of the esophageal adenocarcinoma exhibit a high overexpression of p53.(9,10)

The overexpression of p53 noticed in the cases analysed by immunofluorescence is correlated with the prognosis, the degree of differentiation, the degree of proliferation and it is supported by the number of immunomarked cells and the intensity of the marking.

Genomic DNA was isolated from the patients with esophageal cancer. Total DNA was extracted using the *Wizard Genomic DNA Purification Kit* from Promega.

The purified PCR products were sequenced with the help of the ABI 3130 (Applied Biosystems) genetic analyzer. The sequences obtained both from the samples of tumoral tissue and blood were compared with the p53 sequence from the GenBank database. Numerous nucleotidic polymorphisms were identified. Out of the thirty patients under study, four (40%) presented polymorphisms in p53 gene.

A polymorphism at exon 5 was found in patient 7. This polymorphism corresponds to a heterozygous missense point mutation located at codon 175 (CGCCAC). The normal codon codes for arginine, the modified codon codes for histidine.

Two heterozygous point mutations were identified in exon 6: i) a silent mutation at codon 213 CGACGG; this mutation was found both in the tumoral sample and in the blood of patient 6; the amino acid coded by both codons was arginine; ii) a missense mutation at codon 220 TATTGT in the sample of tumoral tissue of patient 11, codon TAT codes for tyrosine, and codon TGT for cysteine. Cysteine and tyrosine are neutral amino acids, however cysteine is a weakly polarized amino acid, whereas tyrosine is not. This last modification identified at exon 6 can influence the polarity of the protein.

The correlation of the genetic data with the clinical data of the patients shows that the modifications in the p53 gene occur during the early stages (I-II) of esophageal squamous cell cancer. The more advanced stages of cancer can be associated with changes in the polarity of the protein which may lead to modifications of the protein conformation and to the alteration of its response capacity at the level of the tumour.

Analysis of the possible duplications or deletions occurring in the patients diagnosed with esophageal cancer by using the MLPA technique

Modifications of the number of copies occur frequently in the chromosomal sequence, a fact which determines the predisposition of the human body to various syndromes and diseases. The deletion or duplication of one or more exons at the level of reference genes, such as BRCA2 gene, predispose to neoplastic diseases as well.(11)

The MLPA technique for the investigation of the possible correlations between the previously selected gene, BRCA2 and the presence of esophageal cancer

The BRCA2 gene belongs to the class of genes known as tumour suppressors. The protein of the BRCA2 gene helps to prevent cell growth and accelerated or uncontrolled division. BRCA2 is also involved in the DNA repair processes as it plays an important role in maintaining the stability of the genetic information of the cell.

Multiplex Ligation-dependent Probe Amplification (MLPA) is a method developed in order to determine the number of copies for up to 50 sequences of genomic DNA in a single reaction based on multiplex type PCR. MLPA is a method of analysis of the multiplex type of the number of DNA copies used to identify large size mutations in clinical and research investigations.(12)

A number of genes have proved to be involved in the development, progress and response to treatment of esophageal cancer. The phenotypic uniqueness and, consequently, the heterogeneity of the clinical behavior of the tumours of different patients can be followed by determining the variation of the number of copies at the level of those genes.(13) Among these, BRCA2 is one of the most important tumour suppressor genes.

BRCA2 gene (Breast Cancer 2 susceptibility protein) (13q12-13) belongs to the family of tumour suppressor genes (14), (15) and codes a nuclear phosphoprotein which is made up of 3.418 aminoacids.(16,17,18)

The Principle of the Method

DNA was extracted from the tumoral tissue samples and from the leukocytes (for the witness) using the *Wizard® Genomic DNA Purification kit* (Promega). Unlike the isolation using the classical method with chloroform/ isoamyl alcohol, this kit contains all the necessary solutions for the rapid and easy purification of DNA (genomic, mitochondrial, viral) from a wide range of tissues, cell cultures, blood, yeasts and bacteria, based on a saline extraction process in four stages.

The results obtained from the analysis of the deletions using the mlpa technique

A total of 30 patients were investigated using the MLPA technique to show the possible correlations between the chromosomal mutations existing at the level of the BRCA2 gene (deletions or amplifications) and esophageal cancer.

The analysis of the results of MLPA starts from the unprocessed genetic profile resulting after the migration and separation of the amplification products on the ABI Prism 310 genetic analyzer. By processing it with the help of the „GeneMapper ID v3.1” programme, the genetic fingerprint of the people shall be obtained in the end.

All the 30 patients presented at least one modification such as a deletion or an amplification at the level of the BRCA2 gene. A possible correlation between the existence of some deletions at the level of exons 4 and 5 and esophageal cancer was noticed, as this modification was present in all the 30 patients included in the study. Other deletions possibly correlated with the development of esophageal cancer were noticed at the level of exons 23, 24 and 27A in 18 patients out of the 30 patients under investigation. The amplifications which

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could be correlated with the presence of esophageal cancer were found at the level of exons 8 and 10 in 18 patients out of the 30 patients under examination.

Conclusions:

Based on the data from the specialty literature, the following molecular markers were selected for the immunofluorescence tests:

- EGFR and HER2/neu as oncogenes;
- p53 and APC as tumour suppressor genes;

In the cases of esophageal squamous cell cancer under study, an overexpression of EGFR și HER2 is obvious, in comparison with the normal tissue samples.

- The patients in whom the overexpression of these types of oncogenes was found may be potential candidates for the cancer treatment with molecular targets (e.g. anti-EGFR medication).

The evaluation methods of immunofluorescence, of the gene expression, can be useful for the determination and/or confirmation of the diagnosis of the tumours present at the level of the GI tract and, at the same time, they can be used for the selection and orientation of adequate treatment strategies.

The mutations in the p53 gene (polymorphisms, deletions) are among the most common genetic modifications found in human cancers. In the patients who were examined, point mutations were identified at exon 5, codon 175, at exon 6, codons 213 and 220 and at intron 7 in positions +18437, T/C and +18457, T/G.

The MLPA technique was used to investigate the possible correlations between the previously selected gene, BRCA2, and the presence of esophageal cancer. As a result of the investigations, certain regions belonging to these genes which could be correlated with esophageal cancer were discovered.

This refers to the existence of some deletions at the level of exons 4 and 5 belonging to the BRCA2 gene found in all the 5 patients investigated for this gene, of some deletions at the level of exons 23, 24 and 27A found in 18 out of the 30 investigated patients and of some amplifications at the level of exons 8 and 10 in 18 patients.

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