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Prolonged survival after neoadjuvant chemotherapy related with specific molecular alterations in the patients with nonsmall-cell lung carcinoma

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ABSTRACT

Lung cancer is the most common cause of neoplasia-related death worldwide. Accounting for approximately 80% of all lung carcinomas, the non-small cell lung carcinoma (NSCLC) is the most common clinical form with its two predominant histological types, adenocarcinoma (ADC) and squamous cell carcinoma (SCC). Although surgical resection is the most favorable treatment for patients with NSCLC, relapse is still high, so neoadjuvant chemotherapy (NAC) is an accepted treatment modality. In this study we examined whether some of the key molecules associated with the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling pathways could have predictive and prognostic value for the NAC application. To that end we examined the expression status of PTEN, pAKT, pERK and loss of heterozygosity (LOH) of *PTEN* in two groups of NSCLC patients, those who received and those who did not receive NAC. LOH *PTEN* and low pERK expression is shown to be correlated with the longest survival of patients with SCC and ADC, respectively, who received NAC. These results point that the application of NAC is beneficial in the NSCLC patients with specific molecular alterations which could further help to improve constant search for the druggable molecular targets used in personalized therapy.

1. Introduction

Lung cancer is the most common cause of neoplasia-related death worldwide with a variety of histological types. The non-small cell lung carcinoma (NSCLC), which predominantly comprises of adenocarcinoma (ADC) and squamous cell carcinoma (SCC), is the most common histological subgroup of lung cancer, accounting for approximately 80% of all lung carcinomas. Surgical resection is the most favorable treatment for early-stage NSCLC, although relapse is still high, especially in stages II and III (Win et al., 2008). Management of locally advanced NSCLC represents a challenge, particularly because the role of surgery in this group is controversial in view of the fact that most patients relapse within 3 years from diagnosis, causing 5-year overall survival <10%. Considering the high rate of extrapulmonary relapses, possibly due to the presence of distant micro-metastases at the time of diagnosis, neoadjuvant chemotherapy (NAC) is an accepted treatment modality for patients with NSCLC. Potential disadvantages include the development of medical illness and treatment-related toxicity (Boudaya et al., 2013). Therefore, there is a need for the identification of appropriate molecular markers which would identify patients who would benefit from neoadjuvant chemotherapy.

The PI3K/AKT/mammalian target of rapamycin (mTOR) (PI3K) and the RAS/RAF/MAP kinase–ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) (MAPK) pathways are frequently deregulated in human cancers, as well as lung cancer, mostly as a result of genetic alterations in their components (De Luca et al., 2012). PI3K/AKT/mTOR signaling pathway has been shown to be involved in the regulation of cell proliferation and apoptosis, and is a key to the initiation and progression of malignancies, enhancing cell survival by the stimulation of cell proliferation and the inhibition of apoptosis (Cantrell, 2001). The main regulator of this pathway, PTEN (phosphatase and tensin homolog deleted on chromosome 10), frequently altered in lung cancer, reduces the downstream activity of AKT, thereby inducing cell-cycle arrest and apoptosis (Hosoya et al., 1999). Another signaling pathway very important in carcinogenesis is RAS/RAF/MEK/ERK and it promotes cell proliferation, angiogenesis, cell differentiation and migration (McKay and Morrison, 2007).

In the current study we investigated the expression status of PTEN, pAKT, pERK and loss of heterozygosity (LOH) of *PTEN* in two groups of NSCLC patients, those who received and those who did not receive NAC. The aim was to evaluate whether these key components of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling pathways could have the predictive and prognostic value for the rational application of NAC.

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2. Materials and methods

2.1. Tissue samples

Paired samples from cancer and adjacent normal lung tissue from 70 patients with NSCLC who underwent surgery, lobectomy or pneumonectomy with regional lymphadenectomy, at Clinic of Thoracic Surgery, Clinical Centre of Serbia, Belgrade, Serbia, were analyzed. Patients were divided into two groups. One group of 35 patients received NAC, while the other did not. All chemotherapy protocols were platinum-based ranging from I to IV cycles. The samples were collected and used after obtaining an informed consent and approval from the Ethics Committee, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The tumors were histologically classified according to the latest World Health Organization classification of the lung cancer (Travis et al., 2004) while the pathological stage of the tumors diagnosed before 2010 was reclassified according to the recommendation from 2009 (Travis, 2009; Vallières et al., 2009). Diagnosis of NSCLC, the histological grade and regional lymph node involvement were established by histological examinations of the surgical specimens. The patients' distribution by clinicopathologic parameters is presented in Table 1.

2.2. DNA extraction

Specimens obtained from the patients without NAC were frozen in liquid nitrogen, where they were kept until DNA extraction. This DNA was extracted using the phenol/chloroform/isoamyl alcohol method (Sambrook et al., 1989). DNA from paraffin-embedded tumor material from patients with NAC was extracted using a Kappa Express Extract DNA extraction kit (KapaBiosystems, USA) according to the manufacturer's procedure. The concentrations of isolated nucleic acids were assessed spectrophotometrically. The quality of DNA was verified by electrophoresis.

Table 1
Clinicopathological parameters of patients with and without NAC.

Parameter	Patients with NAC	Patients without NAC
Total	35	35
Age		
≤50	6	11
>50	29	24
Gender		
Male	26	23
Female	9	12
NSCLC subtype		
Adenocarcinoma	16	15
Squamous cell carcinoma	19	20
Necrosis[a]		
n1	27	25
n2	5	6
n3	3	4
Inflammation		
Level 1	9	15
Level 2	20	17
Level 3	6	3
Histological grade[b]		
g1	10	6
g2	15	24
g3	10	5
Stage		
I	5	2
II	15	12
III	15	21
Lymph node invasion		
Positive	25	29
Negative	9	6

NAC — neoadjuvant chemotherapy.
[a] n1, obscure or no necrosis; n2, necrosis in 50% of tumor mass; n3, necrosis in more than 50% of tumor mass.
[b] g1, well differentiated; g2, moderately differentiated; g3, poorly differentiated.

2.3. LOH analysis

The DNA obtained from the malignant and normal lung tissue from all 70 patients was used to study the LOH of *PTEN*, tumor suppressor gene. LOH analyses were performed using highly polymorphic microsatellite markers. Five polymorphic microsatellite markers spanning the *PTEN* gene (D10S579, D10S1765, D10S215, AFM086wg9, and D10S541) were selected to cover deletions at the whole *PTEN* locus on chromosome 10q23. All forward primers were 5′-labeled with Fam, Vic, Ned, Pet, and Fam fluorescent dyes, respectively. The choice of the microsatellite markers and locus-specific PCR conditions were determined from published sources (Feilotter et al., 1998; Hahn et al., 1999). The PCR products were separated by capillary electrophoresis on an ABI Prism 3130 automated sequencer and sized using GeneScan-500 LIZ size standard (Applied Biosystems). The obtained data were analyzed with the GeneMapper software (Applied Biosystems). The DNA from normal lung tissue adjacent to tumors from the same patient was used as reference. On the one hand, a marker was defined as noninformative (homozygote) when only 1 allelic peak was detected in the DNA sample of the normal lung tissue. On the other hand, a marker was considered informative (heterozygote) when 2 major allelic peaks occurred in a normal specimen. The LOH score for the informative cases was calculated automatically by GeneMapper software according to the following equation: (peak height of normal allele 2)/(peak height of normal allele 1) divided by (peak height of tumor allele 2)/(peak height of tumor allele 1). A sample was considered to be an LOH candidate for particular locus if the ratio values were less than 0.66 and higher than 1.5.

2.4. Immunohistochemistry

Tumor samples were fixed in buffered 10% formalin, embedded in paraffin blocks and cut in 3 μm for routine analysis. The following antibodies were used according to manufacturer's instructions: PTEN (1:50, clone: PN37, Invitrogen, USA), pERK (1:100, clone: P44/42-MAPL-ERK1/2(137 FS), Cell Signaling, USA) and pAKT (1:40, clone: HCL-l AKT-Phos, Novocastra, Leica Biosystems, USA). Immunostaining was performed by incubating tissue sections with appropriate serum for 30 min at room temperature in humidity chamber, using the streptavidinbiotin technique (LSAB + Kit, Peroxidase Labeling, K0690, DAKO Cytomation, Denmark). Antigen–antibody complexes were visualized with diaminobenzidinehydrochloride (DAB, No. K3468, DAKO Cytomation, Denmark) substrate solution. The cell nuclei were contra-stained with Mayer's hematoxylin. At the same time, tissue samples with appropriate positive immunostaining were used as indicators of the quality of the target retrieval procedure. Positive immunoreactivity of pAKT in epidermis of human skin and PTEN and pERK in regular breast ductal epithelia was used as the internal positive control.

Immunohistochemical (IHC) results were independently evaluated by two pathologists (J.S. and Z.M.) on microscope Leica DM2500 (Leica Microsystems, Germany). The immunoreactivity of PTEN was assessed using the semiquantitative method based on the score of percentage of stained cells—cytoplasm/nuclei (P) (0, no immunoreactivity; 1,1–10%; 2, 11–50%; 3, 51–100%) and intensity of staining (SI) (0, no immunoreactivity; 1, reduced staining intensity relative to the corresponding normal cells; 2, same as normal cells staining; 3, increased staining). Since cutoff levels for reduced PTEN expression by immunohistochemical methods have not been defined so far, we used the mean PTEN score as a cutoff point to designate reduced expression (Shoman et al., 2005). Accordingly, PTEN status was defined as follows: low expression if score was ≤4; and high expression if score was >4. The pAKT staining was evaluated by an H-score, which was calculated