Article is available online at http://www.webio.hu/por/2007/13/2/0099

ARTICLE

Phenotype of Bone Metastases of Non-Small Cell Lung Cancer: Epidermal Growth Factor Receptor Expression and K-RAS Mutational Status

Gayane BADALIAN,[1] *Tamás BARBAI,[2] Erzsébet RÁSÓ,[2] Katalin DERECSKEI,[2] Miklós SZENDRŐI,[1] József TÍMÁR[2]

[1]Departments of Orthopedics, Semmelweis University, [2]Department of Tumor Progression, National Institute of Oncology, Budapest, Hungary

Bone metastasis is a frequent complication of lung cancer progression, however, studies on bone metastatic tissues are scanty. Here we have collected a small cohort of 11 non-small cell lung cancer cases where primary tumors and corresponding bone metastases were available for pathological analysis. We have tested two molecular markers: EGFR protein expression and K-RAS mutation at codon 12 using immunohistochemistry and RFLP-PCR, respectively. We have shown that using improved protocols, EGFR protein (both the extracellular as well as the cytoplasmic domain) is readily detectable in decalcified bone tissue. We found that the EGFR expression status is highly similar in bone metastases compared to the primary tumors, although the expression levels may change. Individual comparison of corresponding primary and metastatic NSCLC tissues indicated that downregulation of EGFR was a rare event (2/11) compared to upregulation (4/11) in bone metastases. On the other hand, our data indicate that the K-RAS mutational status of the primary tumor does not predict the status of the bone metastatic tissue of NSCLC, since we have observed both emergence of mutant clones in metastases from wild-type (wt) primary tumors and loss of mutant clones in metastases from mutant primaries in addition to the maintained mutant status. Our data support that at least two progression models occur in NSCLC, the same-gene as well as the clonal selection one. It is noteworthy that in NSCLC cases with wt- or mutant K-RAS, downregulation of EGFR expression was a rare event although upregulation in bone metastases was observed more frequently in wt K-RAS cases.(Pathology Oncology Research Vol 13, No 2, 99–104)

Key words: EGFR immunohistochemistry, K-RAS mutation, NSCLC, bone metastasis

Introduction

Lung cancer is one of the most aggressive human cancers expressing extreme hematogenous dissemination potential. The bone is frequently involved in lung cancer progression and there is no difference between the histological subtypes in this respect. The underlying molecular mechanisms of the development of bone metastasis is better known in case of breast- or prostate cancer,[1,2] but is less obvious concerning lung cancer. Genomic profiling of lung cancer revealed that squamous cell cancer (SQCC) is characterized by overexpression of several members of the arachidonic acid metabolizing system (LTB4 dehydrogenase and COX-2) which produce bone regulatory prostanoid metabolites.[3] It is worth to mention that PTHrP or bone matrix protein, characterizing other bone metastatic cancer types, is not expressed by SQCC lung cancer. Expression profiling of adenocarcinomas (AC) of the lung revealed that beside the prostanoid synthesizing enzymes this cancer type expresses BMP2 bone matrix protein, probably contributing to the organ preference potential.[4] In small cell lung cancer, expression profiling did not reveal genes that could be linked to bone preference.

Received: Jan 11, 2007; *accepted:* May 10, 2007

Correspondence: József TÍMÁR, MD, PhD, DSc, Department of Tumor Progression, National Institute of Oncology, Ráth György u. 7-9, Budapest, H-1122, Hungary. Phone: 36-1-224-8786, Fax: 36-1-224-8706, E-mail: jtimar@oncol.hu

**equal contribution of the two authors*

Non-small cell lung cancers (NSCLC) are characterized by EGFR overexpression partly due to gene amplification and in a subset of ACs by tyrosine kinase (TK) domain mutations.[5,6] On the other hand, smoking induces K-RAS mutation in 20-30% of NSCLC, more frequently in AC than in SQCC.[7] It has been observed that the K-RAS and EGFR-TK mutations are mutually exclusive.[6,8] EGFR overexpression in NSCLC has no prognostic importance whereas EGFR amplification and especially TK mutations predict favorable response to EGFR-targeted therapies.[9] On the other hand, K-RAS mutation was reported to be a negative prognostic and predictive factor for NSCLC, although other studies challenged this view.[7] It is important to note that almost all of the aforementioned studies on EGFR and K-RAS were performed on primary tumors while much less information is available concerning the metastatic tissue. Therefore we selected a small cohort of bone metastatic NSCLC and compared their EGFR protein expression and K-RAS mutational status to those of the primary tumor. Our data suggest that while the EGFR expression of NSCLC is rather stable during progression to bone, the K-RAS mutational status is more instable, most probably sensitively reflecting clonal changes in the tumor cell population.

Materials and Methods

Patients

We have selected 11 NSCLC cases for this retrospective histopathological analysis from the archive of the Department of Orthopedics with the approval of the local ethical committee. NSCLC patients presented with bone metastases which were at least partially removed by surgery (patients' data are shown in *Table 1*). Paraffin-embedded tumor samples of both the bone metastases as well as primaries were available for analysis.

EGFR immunohistochemistry

Tissue samples were routinely fixed in 10% (v/v) neutral buffered formalin, dehydrated in a graded series of alcohol and xylene and embedded in paraffin at a temperature not exceeding 60ºC. Three to four micron sections were mounted on Superfrost slides (Shandon) and manually deparaffinized. We have used proteinase K antigen retrieval suggested by the manual of EGFR pharmDx™ kit (DAKO, Glostrup, Denmark). Alternatively, slides were immersed in 0.05 mM citrate buffer (pH=6), and exposed to 750 W microwave for 3x5 min (MFX-800-3 automatic microwave, Meditest, Budapest, Hungary).[10] Endogenous peroxidase activity was blocked by 3% H_2O_2 for 5 min at room temperature.

The extracellular domain of EGFR was detected by the EGFR pharmDx™ kit using mouse monoclonal anti-human EGFR (EGFR-EC, clone 2-18C9), dextran polymer conjugated with HRP and goat anti-mouse IgG and DAB substrate-chromogen, applied rigorously following the manufacturer's instructions. As positive controls, slides provided by the manufacturer (formalin-fixed and paraffin-embedded pellet of HT29 human colorectal carcinoma cell line) as well as human head and neck carcinoma tissue sample previously diagnosed 3+ in all tumor cells for membrane EGFR by using EGFR pharmDx™ and CONFIRM anti-EGFR (Ventana) were used.

To detect the cytoplasmic domain of EGFR (EGFR-CY), we used rabbit polyclonal antibody PU355-UP (Biogenex) without dilution. In case of negative control, slides were exposed to the diluent instead of the primary antibody, but were processed in the same way as other slides.

Reactions were developed by an LSAB Kit (DAKO). Immunoreaction was visualized by using diaminobenzidine (DAB) as chromogen. Nuclei were stained by hematoxylin.

Table 1. **NSCLC patients' characteristics**

Case no.	*Sex*	*Age (years)*	*Histology of primary*	*Adjuvant therapy*	*Primary survival (months)*	*Bone metastasis*	*Metastasis therapy*	*Overall survival (months)*
1	F	53	AC	NA	NA	Fe,l	TEP	28
2	M	76	AC	S	18	Fe,l	TEP	36
3	M	67	SQCC	S+CY	35	T,r	PR	42
4	M	57	SQCC	S+CPD, DOXO	31	Fe,r	PR	47
5	F	47	AC	S+CPD	51	Fe,l	TEP	75
6	M	49	AC	S+DOXO	41	Fe,r	PR	59
7	M	72	AC	S+DOXO	46	Fe,r	TEP	55
8	M	66	AC	S+chemo	19	U,r	PR	21
9	M	56	AC	S	25	Fe,r	PR	38
10	M	53	AC	S+DOXO	49	Fe,l	TEP	42
11	F	60	AC	S+CPD	11	Fe,l	TEP	24

F: female, M: male, AC: adenocarcinoma, SQCC: squamous cell carcinoma, S: surgery, CY: cyclophosphamide, CPD: cisplatin, DOXO: doxorubicin, Chemo: undefined chemotherapy, Fe: femoral, T: tibial, U: ulnar metastasis, r: right, l: left, PR: partial resection, TEP: total epiphyseal prosthesis

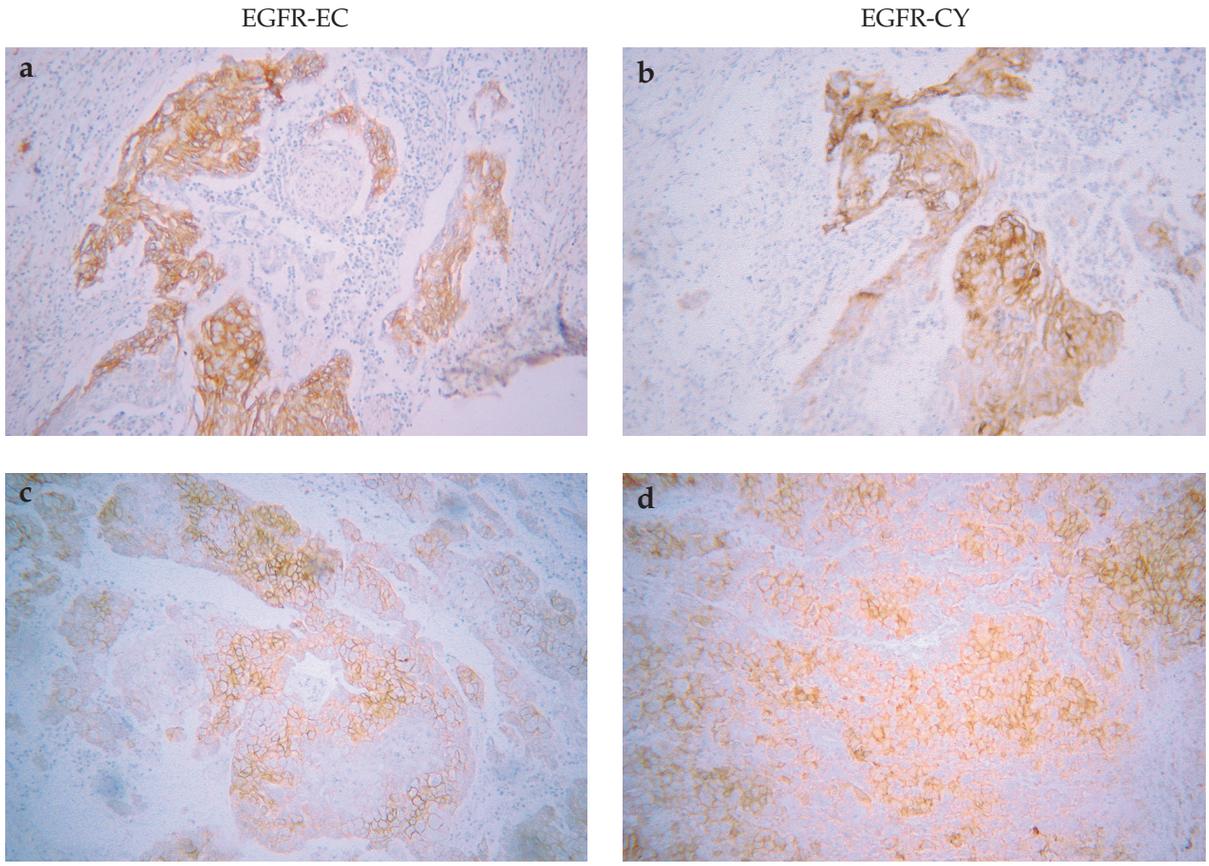

Figure 1. *Detection of EGFR protein in NSCLC samples by immunohistochemistry (Case no. 5).* ***(a,c)*** *Immunoreaction of EGFR-EC domain using EGFR pharmDx™ kit.* ***(b,d)*** *Immunoreaction of EGFR-CY domain using polyclonal anti-C-terminal antibody. a,b: primary tumor, c,d: bone metastasis*

Morphometry

Reactions were evaluated by 2 experts (GB, JT), and the percentage of positive cells as well as the intensity of the reaction (0, 1+, 2+, 3+) were determined in at least 3 areas of the tumor. Mean levels were determined for each tumor and a final IHC score was produced by multiplying the percentage data with intensity values. Statistical analysis was performed using MS Excel program applying t test. Up- or downregulation of expression was defined as >30% alteration in EGFR score confirmed by both antibodies.

PCR-RFLP analysis for K-RAS gene point mutations in codon 12

DNA was extracted from formalin-fixed and paraffin-embedded tissue using the MasterPure™ DNA Purification Kit according to the instructions of the manufacturer. Two primer pairs (nested PCR) were used. DNA amplifications were performed with DyNAzyme™ and Mastercycler gradient thermal cycler supplied by Eppendorf. The reaction mixture of reagents for samples was prepared, containing 2.5 µl 10x PCR puffer+Mg^{2+} (DyNazyme™), 200 µM/each dNTP, 1.00 pM/reaction of each primer, 0.8 U of DyNAzyme™ polymerase per reaction in the first step and 0.25 U DyNAzyme™ polymerase per reaction in the nested step. The inner sense primer was a mismatch primer, and the product of PCR contained the recognition site of BstNI restriction endonuclease in the wild type of K-RAS gene. Outer primer pair: 5'-GCCTGCTGAAAATGACTGAAT-3' and 5'- GGTCCTGCACCAGTAATATG-3', inner primer pair: 5'-GAATATAAACTTGTGGTAGTTGGACCT-3'and 5'-GGTCCTGCACCAGTAATATG -3'. Both pairs had 35 cycles of denaturation at 95ºC for 1 min, primer annealing at 55ºC for 1 min, chain elongation at 72ºC for 2 min. The amplified products were digested with BstNI (New England BioLabs). Enzymatic digestions were performed at 60ºC for 3 h in a total volume of 30 µL. Digested PCR product were separated on 4% agarose gel in TAE buffer and visualized under UV light following ethidium bromide staining.

Results

The majority of the NSCLC patients were male (8/11) and adenocarcinomas (9) dominated over squamous cell cancer (2) (*Table 1*). Except for one case, bone metastases developed following surgery of the primary tumor.

Immunohistochemistry of EGFR expression in primary and bone metastatic NSCLC indicated that similarly to the primary tumors, in bone metastatic (decalcified) cancer tissues EGFR protein could be detected by the EGFR pharmDx™ kit but microwave antigen retrieval and extended incubation with the primary antibody improved the reactions considerably without increasing the nonspecific background (*Fig. 1a,c*).[10] Furthermore, the C-terminal domain of the EGFR was also reliably detectable at both locations (*Fig. 1b,d*).

Morphometric analysis of EGFR expression in the primary tumors compared to the bone metastases demonstrated no significant difference in the expression level as detected by either anti-EGFR antibodies (*Fig. 2*). On the other hand, case by case comparison of the EGFR expression levels in primary and metastatic NSCLC indicated that 45.5% of cases maintained EGFR expression levels, 36.4% demonstrated an increase in bone metastasis, while in 18.2% of cases a significant downregulation was detected (*Table 2*).

All the NSCLC tumor samples contained predominantly (>50% of the sample) tumor tissue in the analyzed paraffin blocks. RFLP-PCR analysis of the paraffin-embedded tumor tissues for K-RAS codon-12 mutation demonstrated that 5 out of 11 NSCLC cases (45.5%) were mutant (*Table 3*). However, primary and metastatic tumors showed a great variability in respect of K-RAS mutation status which was rarely maintained (1/5 cases), but was different in most cases (4/5). It is of importance that the frequency of loss of K-RAS mutation or acquisition of this genotype in bone metastases occurred with equal frequency (2 cases each, *Table 3*).

When the EGFR protein expression changes were compared to the alterations in K-RAS mutation status of the metastatic lesion, it was found that in case of tumors carrying wild-type (wt) K-RAS, EGFR expression was upregulated in metastases in 3 out of 6 cases (50%), while only one out of 6 was downregulated (16.7%, *Table 4*). On the other hand, in 3 out of 5 mutant K-RAS cases the EGFR status was maintained in the metastases (60%), while up- or downregulation was a rare event (one case each, *Table 4*).

Discussion

Lung cancer was long thought to be relatively resistant to chemotherapy, therefore, it was treated mainly by surgery and radiotherapy. However, this trend changed in the past decade with the development of clinically effective chemotherapeutic protocols.[5,11] While the pharmacogenomics of chemosensitivity of lung cancer is still unable to provide highly efficient predictive markers, identification of certain genes might change this trend.[12] On the other hand, in recent years EGFR emerged as a useful target in lung cancer[5,6] and EGFR-targeting agents (mostly small molecular TK inhibitors) presented unprecedented success treating NSCLC where the role of predictive pathology is yet ill-defined.[13]

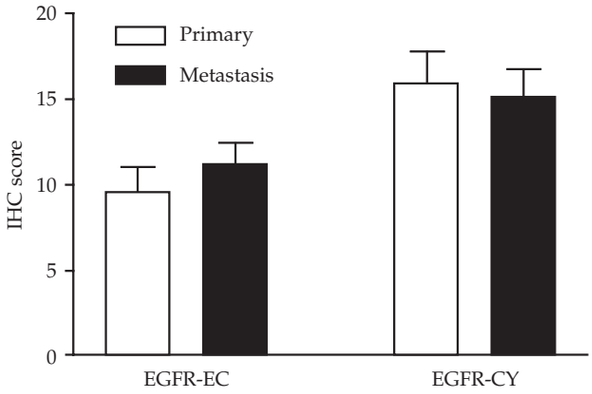

Figure 2. *Comparison of EGFR protein expression of NSCLC in primary and bone metastatic cases (n=11). Data are EGFR intensity scores, expressed as mean±SE and derived from Table 2. EGFR-EC = extracellular domain, EGFR-CY = cytoplasmic domain*

Table 2. **EGFR protein expression in primary tumors and bone metastases of NSCLC**

Case no.	*EGFR-EC*		*EGFR-CY*		*EGFR status in metastasis*
	primary	*metastasis*	*primary*	*metastasis*	
1	10.0	17.0	19.25	23.88	maintained
2	19.76	17.0	23.88	18.0	maintained
3	17.0	10.0	24.88	6.25	downregulated
4	6.88	14.0	12.5	18.0	upregulated
5	13.0	4.32	16.88	9.0	downregulated
6	7.25	12.75	8.8	13.75	upregulated
7	5.88	13.0	12.0	18.8	upregulated
8	7.0	5.0	8.0	17.5	maintained
9	8.0	11.25	24.88	14.88	maintained
10	6.0	9.25	14.0	18.5	upregulated
11	4.88	8.63	11.5	7.25	maintained
				EGFR maintained	5/11 (45.5%)
				EGFR upregulated	4/11 (36.4%)
				EGFR downregulated	2/11 (18.2%)

Data are expressed as mean of IHC score. Maintained = no change in IHC score in metastasis, or equivocal results with the two antibodies; downregulated = decrease of IHC score by >30%; upregulated = increase in IHC score by >30%

Table 3. **K-RAS mutational status of NSCLC**

Case no.	*K-RAS status*		
	primary	*metastasis*	*NSCLC case*
1	wt	M	mutant
2	wt	wt	wt
3	wt	wt	wt
4	wt	wt	wt
5	wt	M	mutant
6	M	M	mutant
7	wt	wt	wt
8	wt	wt	wt
9	M	wt	mutant
10	wt	wt	wt
11	M	wt	mutant
K-RAS mutation	3/11 (27.3%)	3/11 (27.3%)	5/11 (45.5%)

wt= wild-type, M= mutated at codon 12

There are three major models of tumor progression/metastatization: clonal evolution/selection,[14] parallel development[15] and the same-gene models,[16] providing sharply different explanations for tumor progression. The two extremes are the parallel development and the same-gene models, where the former predicts very early generation of disseminated cancer cells to distant organs with highly diverse genetic profiles of the primary and metastasis whereas the latter suggests metastasis as a relatively late event of tumor progression, therefore genetic diversity of the metastases is suggested to be minimal.[15,16] The clinical validity of these models can only be determined by direct comparison of metastatic and primary tumor tissues of various cancer types. Concerning the progression of lung cancer it seems that clinicopathological data support all the three metastasis models when using LOH,[17] CGH[18] and SNP techniques[19] on metastatic and primary lung tumor tissues, although the cohort sizes were small (<10 cases).

Table 4. **Comparison of EGFR protein expression and K-RAS mutation changes in bone metastases of lung cancers**

Case no.	*KRAS change*	*EGFR change*
2	wt maintained	maintained
3	wt maintained	downregulated
4	wt maintained	upregulated
7	wt maintained	upregulated
8	wt maintained	maintained
10	wt maintained	upregulated
1	Mutated in metastasis	maintained
5	Mutated in metastasis	downregulated
6	Mutated in primary and met.	upregulated
9	Mutated in primary	maintained
11	Mutated in primary	maintained

Maintained, up- or downregulated: see Table 2 for definition

Bone is among the most frequent sites for metastasis of lung cancer, but pathologic studies on bone metastatic disease or on the metastatic tissues are surprisingly rare in the literature. Therefore, we have collected a small cohort of bone metastatic NSCLC cases where both the primary and the metastatic tissues were available for analysis. Since EGFR-targeted therapy of NSCLC is applied at advanced organ metastatic stage of the disease, we have studied the EGFR expression profile of the primary tumor and the corresponding bone metastasis. IHC determination of EGFR protein expression is frequently criticized in the literature due to its highly inconsistent results. We have used two commercially available anti-EGFR antibodies for immunodetection of EGFR in bone metastases of NSCLC, recognizing both the extracellular and cytoplasmic domains. Since this tissue has to be decalcified before processing, the sensitivity of the EGFR protein detection was questionable. We have fine-tuned the EGFR pharmDx™ protocol to improve the sensitivity of the detection of the extracellular domain of the protein in the decalcified bone metastatic tissue.[10] Both in primary and bone metastatic tissues the two antibodies provided comparable results on EGFR protein expressions in the individual cases. More interestingly, we found that the expression level of EGFR protein was highly similar in the bone metastasis group compared to that in primary NSCLC. Individual comparison of corresponding primary and metastatic tissues indicated that downregulation of EGFR was a rare event (2/11 cases) while upregulation was observed more frequently (4/11 cases), however, the expression level was maintained in about half of the analyzed cases. It is important to emphasize that positive to negative or negative to positive EGFR conversions did not occur in our small cohort of NSCLC. This observation suggests that EGFR expression status is relatively well-preserved during metastatic progression of NSCLC to the bone, therefore, concerning positivity, the profile determined in the primary tumor is predictive for the (bone) metastatic tissue. Recent studies on brain metastases of lung cancer also found that the mutational status of EGFR and p53 of the primary tumor was preserved.[19,20]

K-RAS is the hallmark of a relatively early genetic aberration during smoking-induced lung carcinogenesis.[7] On the other hand, constitutively active (mutated at codon 12) K-RAS in NSCLC may define a more aggressive and/or drug-resistant genotype.[7] It is also established that EGFR mutations do not occur together with K-RAS mutations.[6,8] However, there are no data on the possible changes in this status during the progression of lung cancer or in bone metastases. Here we have shown data that the K-RAS

codon 12 mutational status of the primary tumor does not predict the status of the bone metastatic tissue of NSCLC, since we have observed both emergence of mutant clones in metastases from primary tumor carrying wild-type (wt) K-RAS and loss of mutant clones in metastases, in addition to the maintained mutant status. These data are not influenced by the amount of tumor tissue in the analyzed materials, since normal tissues were minor components of these samples (<50%). Our data support those views that at least two progression models occur in NSCLC, the same-gene as well as the clonal selection one.[14,16] On the other hand, it is noteworthy that in both wt or mutant K-RAS cases of NSCLC, downregulation of EGFR expression was a rare event, although upregulation in bone metastases was observed more frequently in wt K-RAS cases. These data are based on a small NSCLC cohort and must be confirmed on a larger series of cases.

Acknowledgment

This work is supported by the Ministry of Education (NKFP1a-0024-05).

References

1. *Mundy GR:* Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2:584-593, 2002
2. *Roodman GD:* Mechanisms of bone metastasis. N Engl J Med 350:1655-1664, 2004
3. *Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M, van de Rijn M, Rosen GD, Perou CM, Whyte RI, Altman RB, Brown PO, Botstein D, Petersen I:* Diversity of the gene expression in adenocarcinoma of the lung. Proc Natl Acad Sci USA 98:13784-13789, 2001
4. *Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Thomas DG, Lizyness ML, Kuick R, Hayasaka S, Taylor JM, Iannettoni MD, Orringer MB, Hanash S:* Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nat Med 8:816-824, 2002
5. *Kopper L, Tímár J:* Genomics of lung cancer may change diagnosis, prognosis and therapy. Pathol Oncol Res 11:5-10, 2005
6. *Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F:* EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857-865, 2005
7. *Aviel-Ronen S, Blackhall FH, Shepherd FA, Shepherd FA, Tsao MS:* K-ras mutations in non-small cell lung carcinoma: a review. Clin Lung Cancer 8:30-38, 2006
8. *Soung YH, Lee JW, Kim SY, Seo SH, Park WS, Nam SW, Song SY, Han JH, Park CK, Lee JY, Yoo NJ, Lee SH*: Mutational analysis of EGFR and K-RAS genes in lung adenocarcinomas. Virchows Arch 446:483-488, 2005
9. *Riely GJ, Politi KA, Miller VA, Pao W:* Update on epidermal growth factor receptor mutations in non-small cell lung cancer. Clin Cancer Res 12:7232-7241, 2006
10. *Derecskei K, Moldvay J, Bogos K, Tímár J:* Protocol modifications influence the result of EGF receptor immunodetection by EGFR pharmDx™ in paraffin-embedded cancer tissues. Pathol Oncol Res 12: 243-246, 2006
11. *Molina JR, Adjei AA, Jett JR:* Advances in chemotherapy of non-small cell lung cancer. Chest 130:1211-1219, 2006
12. *Bepler G, Li X, Sharma A et al:* Clinical value of tumoral RRM1 and ERCC1 expressions for treatment response and therapeutic decisions in NSCLC. In: ASCO 2006 Education Book, Ed.: M.C. Perry, ASCO, Alexandria, 2006, pp 431-435
13. *Patel JD:* Epidermal growth factor receptor pathway targeted therapy in patients with aerodigestive malignancies. Curr Opin Oncol 18:609-614, 2006
14. *Fearon ER, Vogelstein B:* A genetic model for colorectal tumorigenesis. Cell 61:759-767, 1990
15. *Gray JW:* Evidence emerges for early metastasis and parallel evolution of primary and metastatic tumors. Cancer Cell 4:4-6, 2003
16. *Bernards R, Weinberg RA:* A progression puzzle. Nature 418:823, 2002
17. *Shiseki M, Kohno T., Nishikawa R, Sameshima Y, Mizoguchi H, Yokota J:* Frequent allelic losses on chromosomes 2q, 18q, and 22q in advanced non small cell lung carcinoma. Cancer Res 54:5643-5648, 1994
18. *Petersen S, Aninat-Mayer M, Schluns K, Gellert K, Dietel M, Petersen I:* Chromosomal alteration in the clonal evolution of the metastatic stage of squamous cell carcinoma of the lung. Br J Cancer 82:65-73, 2000
19. *Takahashi K, Kohno T, Matsumoto S, Nakanishi Y, Arai Y, Yamamoto S, Fujiwara T, Tanaka N, Yokota J:* Clonal and parallel evolution of primary lung cancers and their metastases revealed by molecular dissection of cancer cells. Clin Cancer Res 13:111-120, 2007
20. *Matsumoto S, Takahasi K, Iwakawa R, Matsuno Y, Nakanishi Y, Kohno T, Shimizu E, Yokota J:* Frequent EGFR mutation in brain metastases of lung adenocarcinoma. Int J Cancer 15:1491-1494, 2006