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Application of molecular epidemiological biomarkers by monitoring the effects of treatment in colorectal cancer during follow-up study

Applicazione di biomarkers molecolari epidemiologici mediante il monitoraggio degli effetti del trattamento nel cancro colorettale durante uno studio di follow-up

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Summary

Aim. **We tested the applicability of expression of onco/tumor suppressor genes as biomarkers for monitoring the therapeutical efficacy of colorectal cancer, and compared it with traditional tumor markers (CA19-9 and CEA) and with the total blood antioxidant capacity.** ***Materials and methods.*** **In our follow-up study the expression of oncogenes and tumor suppressor genes of colorectal cancer patients was examined from peripheral blood. The expression of c-myc, Ha-ras, Bcl-2 oncogene and p53 tumor suppressor gene was measured and compared to the level of classical tumor markers, such as CA19-9 and CEA, and the antioxidant capacity in the blood was measured as well. The tests were performed on the day of surgery, on the seventh**

Riassunto

Finalità. **Abbiamo testato se è possibile l'utilizzo dell'espressione di oncogeni o geni soppressori come biomarcatore al fine di monitorare l'efficacia terapeutica su cancro colorettale e paragonarla con i tradizionali marcatori tumorali (CA19-9 e CEA) e la capacità antiossidante ematica totale.** ***Materiali e Metodi.*** **Nel nostro studio di follow-up l'espressione di oncogeni e geni soppressori tumorali dei pazienti con cancro colorettale è stata valutata nel sangue periferico. L'espressione di c-myc, Ha-ras, oncogene Bcl-2 e del gene soppressore tumorale p53 è stata misurata e paragonata al livello di marcatori tumorali classici CA 19-9 e CEA, così come è stata misurata la capacità antiossidante ematica. I test sono stati effettuati lo stesso giorno dell'intervento chi-**

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postoperative day, and after 3, 6, 12 months. Twenty patients, with locally advanced disease took part in the study. Neoadjuvant chemo-radiotherapy was given to the patients according to the onco-surgical guidelines. *Results.* The level of c-myc expression was continuously decreasing after surgery. The other oncogenes and the p53 tumor suppressor gene showed different patterns, but all the examined genes showed significantly lower expression 12 months after surgery. The CA19-9 level showed a decrease during the follow-up, while the CEA concentration was increasing, but both markers presented an elevated level at the 12th month of the study. The changes in the blood antioxidant capacity showed variable values, no correlation with other markers was observed. *Conclusions.* The expression of the studied genes was found to be in a good correlation with the efficacy of the surgery, enabling monitoring the status of CRC patients better than with traditional tumor markers. Eur. J. Oncol., 16 (2), 99-104, 2011

Key words: **molecular epidemiological biomarkers, colorectal cancer, gene expression, chemotherapy**

rurgico, il settimo giorno postoperatorio e dopo 3, 6, 12 mesi. Allo studio hanno preso parte 20 pazienti con malattie avanzate a livello locale. È stata effettuata sui pazienti chemioradioterapia neoadiuvante secondo le linee guida della onco-chirurgia. *Risultati.* Il livello dell'espressione di c-myc è diminuito continuamente dopo l'intervento. Gli altri oncogeni e il gene soppressore p53 hanno mostrato andamenti differenti ma tutti i geni esaminati hanno mostrato una espressione significativamente minore 12 mesi dopo l'intervento. Il livello di CA 19-9 ha mostrato un decremento durante il follow-up, mentre la concentrazione di CEA è aumentata ma entrambi i marcatori presentavano un livello elevato al 12° mese di studio. I cambiamenti nella capacità antiossidante ematica hanno mostrato valori variabili e non è sta osservata nessuna correlazione con altri marcatori. *Conclusioni.* È stata rilevata una buona correlazione tra l'espres-sione dei geni studiati e l'efficacia dell'intervento permettendo il monitoraggio dello stato dei pazienti con cancro colorettale meglio che con i tradizionali marcatori tumorali. Eur. J. Oncol., 16 (2), 99-104, 2011

Parole chiave: **biomarkers molecolari epidemiologici, cancro colorettale, manifestazione di gene, chemioterapia**

Introduction

Colorectal cancer (CRC) mortality is the second highest among malignant tumors (1). Therefore early detection has a high impact on primary, secondary and tertiary prevention of CRC. This is possible through early molecular epidemiological biomarkers, correlating with the disease.

The process of chemical carcinogenesis was well traceable in animal models (developed earlier by our research group) by determining the expressions of Ha-ras, c-myc, Bcl-2, K-ras and p53 genes, well indicating the early biological effects (2, 3). Moreover, these onco/tumor suppressor genes are proven biomarkers of environmental carcinogenic exposure in humans, as well (3). In the present study we wanted to determine whether these molecular epidemiological biomarkers can be used for monitoring the status of colorectal cancer patients undergoing surgical and chemotherapic treatment.

Based on previous studies (4, 5), we supposed, that the effect of the surgery and chemotherapy will be reflected in changes of gene expressions patterns. Chemotherapeutic protocols (as chemical exposure) containing 5-fluoro-uracyl (5FU) and cisplatin increase the expression of onco/tumor suppressor genes, in accordance with the observation that secondary malignancies such as leukemia occurred

in some cases (6, 7). From the point of prevention of secondary tumors, the recognition of changes in these early biomarkers is very important.

On the other side, monitoring CA19-9 and CEA levels is also important in the diagnoses of possible recurrences (8). In our study we compared the levels of tumor markers, tracing the status of carcinomas, with the expression of key onco/tumor suppressor genes to elucidate their ability to forecast or signal the recurrences with better accuracy.

The applicability of the expression of onco/tumor suppressor genes as biomarkers of CRC status in treated patients can be confirmed or disapproved by a long term follow-up study. Is there a good correlation between gene expression changes and the final outcome of disease? When will the level of the studied gene expressions reach the gene expression levels found in healthy populations? Do the level of the classical tumor markers or gene expressions better reflect the status of CRC patients?

Materials and methods

Twenty (10 males and 10 females) colorectal cancer patients between 29 and 81 years of age participated in the study. The group was homogeneously built up with respect to age distribution. The histological diagnosis of diseases was adenocarcinoma, with clinical stage I-III. The patients underwent adjuvant chemotherapy after surgery according to the oncological guidelines (de Gramont protocol) (9).

Peripheral blood was collected from patients for full blood count, determining CEA (IU/ml) and CA19-9 (ng/ml) tumor markers and standard lab parameters (as professional practice ordered), at the day of surgical treatment, on the seventh postoperative day, then in the third, sixth and twelfth month. Leucocytes were isolated by density-gradient centrifugation, tumor markers were measured by ELISA method. For the gene expression studied, total cellular RNA was isolated using TRIZOL reagent (Invitrogen, Paisley, Scotland, UK). The concentration and quality of the RNA was checked by absorption at 260/280 nm wavelength. RNA of each sample (10 μg) was dot-blotted onto Hybond N+ nitrocellulose membranes (ECL kit, Amersham, Little Chalfont, England, UK) and hybridized with chemiluminescently labelled specific probes of c-myc, p53, Bcl-2, K-ras, Ha-ras genes. The RNA isolation, hybridization and detection were performed according to the manufacturer's instructions. The signals were detected on X-ray films, and evaluated by Quantiscan software (Biosoft, Cambridge, UK). Gene expression was related to the level of the constitutively expressed beta actin and the difference was given in percentage (10).

The method of deoxy-ribose degradation test is based on spectrophotometric measurement of thiobarbituric acid (TBA) derivatives of the reactive carbonyl compounds that are formed during degradation of 2-deoxy(-D)-ribose by hydroxyl radicals ($HO^{\cdot}$) generated in the Fenton- reaction between iron(II)- ions and hydrogen peroxide (H_2O_2). In the presence of hydroxyl radicals scavenger substances like antioxidants in the blood, the absorbance of oxidated compounds decreases (11).

Results

The expression of the c-myc gene was continuously decreasing, which was sharper during the second half of the observation period (fig. 1). A decreasing tendency was observed at the p53 tumor suppressor gene, but here the decrease started in the sixth month. The Ha-ras gene had a more or less constant expression, and only at the twelfth month showed significantly lower levels than before. The K-ras and Bcl-2 genes had overall low expression patterns. Altogether, the studied genes had significantly lower expressions in the twelfth month of the follow-up than at the beginning. The surgical therapy (combined with (neo) adjuvant) strongly decreased the expression of c-myc, Ha-ras onco- and p53 suppressor gene at the twelfth month of our investigation.

The CA19-9 level showed a slight increase during the first 3 months, followed by a decrease at the sixth, and a sharp increase at the twelfth month (fig. 2). The CEA level was continuously decreasing, with a sudden peak at the twelfth month (fig. 3).

The antioxidant capacity of blood did not give a clear tendency, however, one week after the therapy it showed a decrease (which could be explained by

the stress caused by the surgery), and a similar decrease was observed in the sixth month (fig. 4).

Discussion

The correlation between the effect of multimodal therapy and the expression of onco/tumor suppressor genes (c-myc, Ha-ras, Bcl-2, K-ras, p53) has not yet been studied. The surgical treatment combined with adjuvant chemotherapy caused a significant decrease in the expression of the studied genes. Based on theoretical considerations and previous studies, the p53 overexpression most probably means a carcinogenic exposure, DNA damage or presumable tumor development. If the expression of the p53 tumor suppressor gene decreases, it is a possible indicator of better tumor – or health – status (apart from a possible loss of function caused by point or chromosome mutation). Continuous decline of c-myc and p53 expression in case of CRC refers to the common and simultaneous participation of these genes in cellular regulatory pathways. The similarity of their trends could refer to special regulatory mechanisms

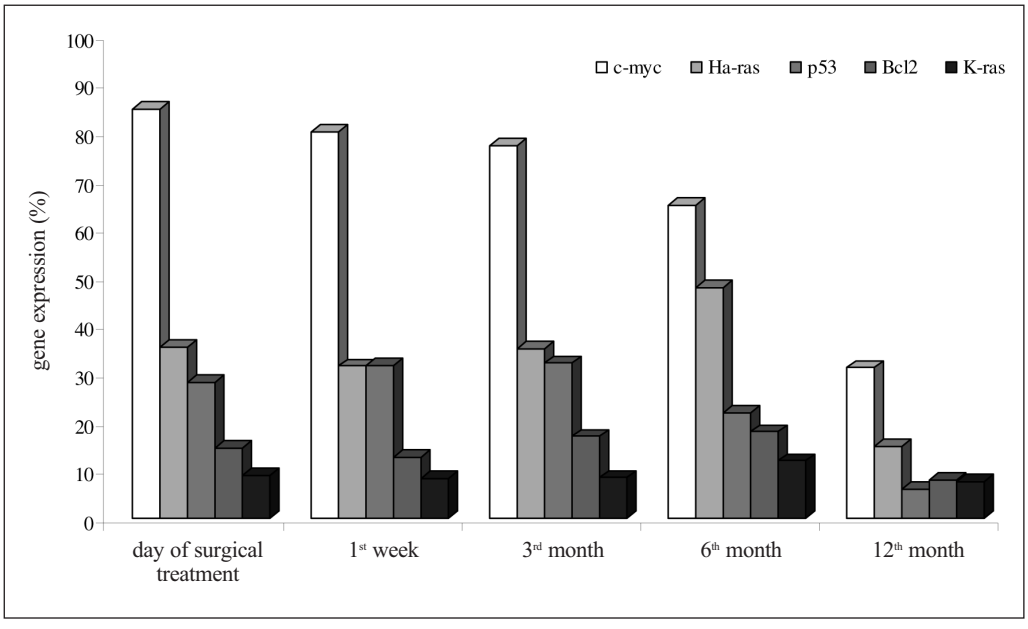

Fig. 1. Gene expression pattern from blood samples of patients group with colorectal cancer (mean)

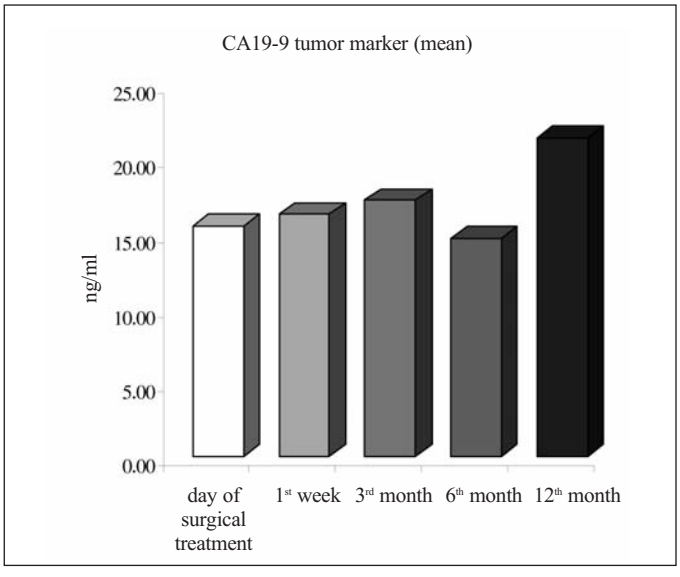

Fig. 2. CA 19-9 tumor marker (ng/ml), from blood samples of patients group with colorectal cancer (mean)

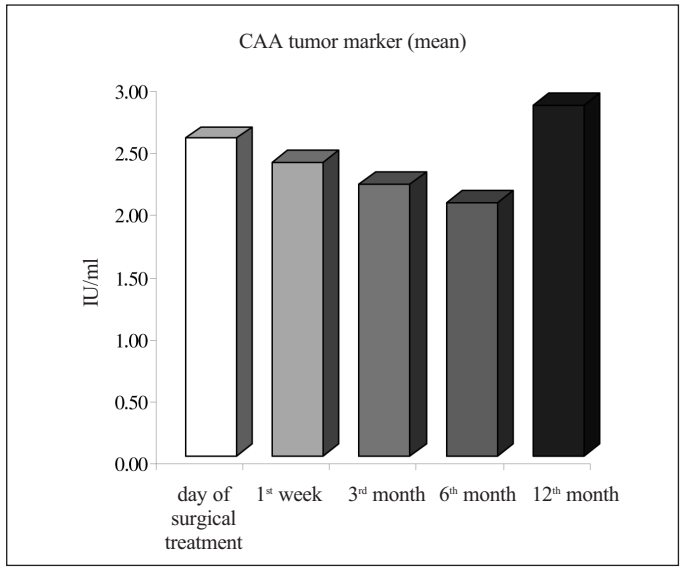

Fig. 3. CEA tumor marker (IU/ml, from blood samples of patients group with colorectal cancer (mean)

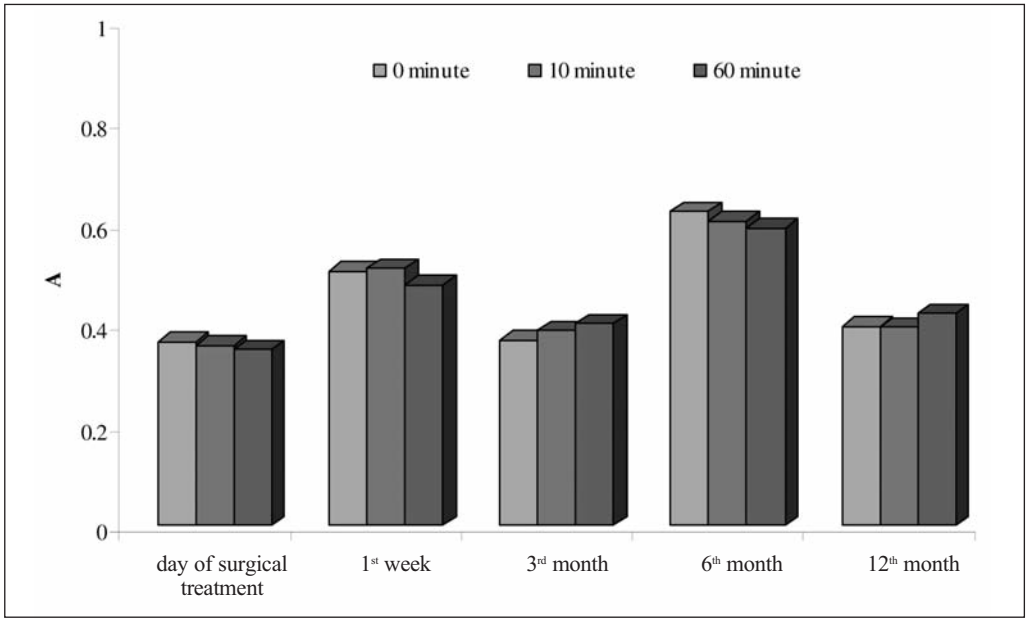

Fig. 4. Absorptions of deoxyribose degradation test after 0, 10 and 60 minutes degradation from blood samples of patients group with colorectal cancer (mean)

of gene expression, for example a hypothetical negative feedback mechanism (12).

The role of Ha-ras oncogene is pregnant in the beginning of tumorigenesis by an activation through point mutations, but it is an important part of the intracellular signal transduction pathways, and it has an important role in later phases of tumor development. In our study, during the follow-up period its level was decreasing, in accordance with the lack of recurrences and distant metastases (6, 7). Since Ha-ras alterations were not found after chemotherapy, consequently we hypothesize that the de Gramont protocol does not induce a Ha-ras overexpression (6, 7).

We should point out that secondary leukaemias induced by chemotherapy typically appear between the second and tenth year after the cytostatic treatment (most common in the fifth year), and the duration of our study was one year (13). Theoretically a Ha-ras overexpression could signalize the formation of a secondary tumor (e.g. leukaemia), and this possibility must be tested in a long term study.

The low expression of c-myc, Ha-ras and p53 at the twelfth month was similar to the gene expression pattern of healthy individuals (2). The expression of these genes is significantly lower in a healthy population than in cancer patients, e.g. CRC (4).

We found in our earlier examinations, that c-myc and Ha-ras expression can be higher in different type of cerebral tumors, as well (14). In the case of small-cell-lung cancer the expression of c-myc was also elevated, measured from peripheral blood leucocytes (15).

The function of K-ras overexpression and activation by mutation is clinically proven in tumor beginning, progression and in the development of distant metastases (16). This observation was in accordance with our findings: the continuously low K-ras expression was associated with the lack of recurrences or metastases.

Overexpression of the antiapoptotic Bcl-2 gene contributes to the immortalization of tumor cells (17). The expression of Bcl-2 remained at low level in our study, well reflecting the recovery stage and the lack of recurrences. All our patients were symptom-free one year after the diagnosis.

The overexpression of molecular epidemic key genes, measured in peripheral blood leucocytes in our study can be related to the presence of the malignant tumor. Their decrease well indicated the changes in the human body, after tumor removal. Application of these markers seems to be a promising way of signalling complications, for example metastases or recurrences, and they can be used for tracing the tumor status.

CA19-9 and CEA tumor markers can refer to residual tumor mass, or presence of tumor recurrence. Their level is in a good correlation of the

existing tumor tissue which is a basis of their widespread use in the clinical practice (8, 18). In our current investigation, however, the serum concentration of these markers did not correlate either with each other (with the exception of the twelfth month), or with the decreasing expressions of the studied onco/tumor suppressor genes. We should observe that the lack of the decrease in the level of CA19-9 markers after the therapy can be partially explained by the fact that the majority of our CRC patients did not have a significantly elevated CA19-9 marker concentration at the time of the surgery. The gene expressions seemed to be more sensitive biomarkers in our present study, giving a better correlation with the therapy and surgical tumor removal.

The antioxidant level in blood seemed to be variable, according to the desoxyribose-degradation test. The antioxidant capacity of the blood, as measured in our study, originates from soluble antioxidant intake, and decreases during oxidative stress. The antioxidant capacity of scavenger type water soluble molecules in the blood did not show any correlation with other biomarkers measured in this study. According to our results, measurement of the antioxidant capacity is not useful in the follow-up of CRC (and its molecular pathways). However, during the week after surgery, a somewhat decreased antioxidant capacity was measured, reflecting the operative stress, and validating the antioxidant capacity as a stress response biomarker.

References

1. Lakatos P, Lakatos L. Current concepts in the genetics of hereditary and sporadic colorectal cancer and the role of genetics in patient management. Orvosi Hetilap 2006; 147: 363-8.
2. Ember I, Kiss I, Raposa T. The usefulness of in vivo gene expression investigations from peripheral white blood cells: a preliminary study. Eur J Cancer Prev 1999; 4: 331-4.
3. Ember I, Kiss I, Gombkötő G, *et al*. Oncogene and suppressor gene expression as a biomarker for ethylene oxide exposure. Cancer Detect Prev 1998; 3: 241-45.
4. Sánchez-Pernaute A, Pérez-Aguirre E, Cerdán FJ, *et al*. Overexpression of c-myc and loss of heterozigosity on 2p, 3p, 5q, 17p and 18q in sporadic colorectal carcinoma. Rev Esp Enferm Dig; 2005; 3: 97.
5. Csontos Z, Nádasi E, Csejtey A, *et al*. Oncogene and tumor suppressor gene expression changes in the peripheral blood leukocytes of patients with colorectal cancer. Tumori 2008; 1: 79-82.
6. Németh Á, Nádasi E, Gyöngyi Z, *et al*. Early effects of different cytostatic protocols for head and neck cancer on oncogene activation in animal experiments. Anticancer Res 2003; 23: 4831-5.
7. Raposa T, Várkonyi J. The relationship between sister chromatid exchange induction and leukemogenicity of different cytostatics. Cancer Detect Prev 1987; 1-2: 141-51.
8. Uehara M, Manaka D, Baba S, *et al*. Prognostic study of preoperative serum levels of CEA and CA 19-9 in colorectal cancer. Gan To Kagaku Ryoho 2007; 9: 1413-7.
9. de Gramont A, Bosset JF, Milan C, *et al*. Randomized trial comparing monthly low-dose leucovorin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J Clin Oncol 1997; 2: 808-15.
10. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinum thiocyanate phenol chloroform extraction. Anal Biochem 1987; 162: 156-9.
11. Rozmer Zs, Perjési P. Effect of certain non-steroid anti-inflammatory agents on the degradation of 2-deoxy-D-ribose initiated by Fenton-reaction. In Hungarian. Acta Pharmaceutica Hungarica 2005; 75: 87-93.
12. Munro AJ, Lain S, Lane DP. P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer 2005; 92 (3): 434-44.
13. van Kaick G, Delorme S. Therapy-induced effects in normal tissue. Radiologe 2008; 48 (9): 871-80.
14. Kiss I, Dezsényi E, Kiss T, *et al*. Detection of elevated oncogene expressions in brain tumours and their macroscopically healthy surrounding tissues. Eur J Cancer Prev 1998; 5: 417-19.
15. Koncz A, Varga I, Kiss I, *et al*. Gene expression changes in peripheral white blood cells of lung cancer patients. In Hungarian. Egészségtudomány 1996; 40: 283-5.
16. Smakman N, Borel Rinkes IH, Voest EE, *et al*. Control of colorectal metastasis formation by K-Ras. Biochim Biophys Acta 2005; 1756 (2): 103-14.
17. Norton JD, Atherton GT. Coupling of cell growth control and apoptosis functions of Id proteins. Mol Cell Biol 1998; 4: 2371-81.
18. Levy M, Visokai V, Lipska L, *et al*. Tumor markers in staging and prognosis of colorectal carcinoma. Neoplasma 2008; 55 (2): 138-42.