Quantitative Estimation of CEA and CK20 Expression in Tumour Tissue of Colorectal Cancer and its Liver Metastases with Reverse Transcription and Real-time PCR

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Abstract. Background: Carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) are established tumour markers and the CEA pre-operative levels in serum have prognostic value. The aim of this study was to verify the usefulness of the estimation of CEA and CK20 gene expressions in tissues of colorectal cancers and their liver metastases. Patients and Methods: Two hundred and twenty-two specimens of colorectal cancers, their liver metastases, other liver tumours and control tissues were analysed by reverse transcription combined with real-time PCR. Results: The expressions of CEA and CK20 were significantly higher in tumours than in controls; there were differences between tumour types and no relationship to staging or clinical development was found. CK20 expression was inversely dependent on grading. The CEA expression in tumours did not correlate with the CEA levels in serum, but did correlate with serum tissue-specific polypeptide antigen (TPS). Conclusion: The measurement of CEA and CK20 gene expressions in tumours did not supply any new prognostic information, but raised the question of the mechanism releasing CEA into the blood.

Colorectal cancer (CRC) is not only the second most common cancer in the Czech Republic among both males and females, but is also one of the most common cancers worldwide, and is accompanied by significant mortality. Despite all the research efforts, the only curative treatment remains surgery and the best prognostic method is tumour staging based on histopathological examination. Multiple biochemical markers have been suggested and tested for screening, aiding diagnosis, determining prognosis and monitoring the treatment of the disease (1, 2).

The carcinoembryonic antigen (CEA or CEACAM 5) holds a special position among all the tumour markers. CEA is a cell-surface glycoprotein, normally expressed in foetal tissue but exhibiting only marginal expression in adults (3). It is a member of the immunoglobulin gene superfamily found on chromosome 19. The function and biological role of CEA is not totally clear, but evidence that it serves as an intercellular adhesion molecule has been presented (4), while its role as an intestine mucosa-protecting molecule has also been documented (3). CEA has been known for almost 4 decades and it is believed that, in normal colon, it is released from the apical surface of polarised columnar epithelial cells into the gut, whereas in dysregulated tumour tissue exfoliated CEA is released into the blood and lymphatic vessels through intercellular spaces. In addition, CEA is overexpressed in many tumours, mainly in CRC. Therefore, in many patients the serum level of CEA rises and pre-operative CEA levels provide independent prognostic information and may also help with surgical management (2).

In contrast to the CEA levels in serum, the significance of the CEA levels in tumour tissue itself remains controversial. Two approaches have been exploited for the estimation of CEA expression in tumour tissue: immunohistochemistry (5, 6) and determination of CEA in the cytosol or even in the pellet of tumour tissue (7-9). Both methods employ specific antibodies to quantify CEA expression. Estimations in cytosols and pellet extracts exploit the standard techniques for serum estimation, while immunohistochemistry requires the subjective grading of an experienced pathologist who evaluates the staining strength and the number of positive cells. In some publications, a higher CEA expression in...
tumour was connected with invasive growth (5), with a higher risk of local recurrence (6) or was correlated with grading (7). In other reports, no correlation with grading (10) or staging (11), or both (12), was observed. One report revealed that the CEA content in the tissue pellet correlated with the CEA level in the serum (13). An absence of correlation between CEA levels in the cytosol and serum was also described, together with the finding that higher CEA levels in the cytosol correlated with better prognosis (14).

Here, we attempted to verify the usefulness of CEA expression measurement in tumour tissue by estimating the levels of specific CEA mRNA using reverse transcription followed by real-time PCR. This technique has been used repeatedly to detect micrometastases in lymph nodes (15-17) or the circulating cells (18, 19) in the blood but, surprisingly, it has not yet been used for the estimation of CEA gene expression in the tumour itself. Another epithelial cell and tumour marker, cytokeratin 20 (CK20) (16, 20, 21), was estimated in parallel.

Patients and Methods

Tissue samples. In this study, the following tissue samples were included: 25 specimens of benign colon and liver tissues (from 15 patients, aged 19.2 - 70.3 years, mean 40.1), 12 specimens of colon mucosa from healthy margins of operated CRC (only the cases with no recurrent disease and no generalization were included) from 12 patients (aged 50.8 - 77.0 years, mean 62.4), 4 specimens of healthy liver tissue (from 2 patients), 85 specimens of CRC tissue of adenocarcinoma type (from 67 patients, aged 28.4 - 87.3 years, mean 64.5), 75 specimens of liver metastases of CRC (from 42 patients, aged 29.4 - 75.6 years, mean 59.1) and 21 specimens of malignant liver tumours other than metastases of CRC (21 patients, aged 39 - 73 years, mean 56.9). All the patients had undergone surgery in the Surgical Clinic of the Faculty Hospital in Pilsen, Czech Republic during the years 1998-2003. Tissue samples were taken during the surgery, frozen to −80°C and kept at this temperature until used. Routine histology examinations of operated tumours were done in the Department of Pathology, Faculty Hospital in Pilsen.

RNA extraction. Total RNA was isolated from 50-100 mg of tissue with the RNAgent Total RNA Isolation System (Promega Corporation, East Port, Prague, Czech Republic). The amount of isolated total RNA was assessed spectrophotometrically and the RNA was kept at −75°C.

Reverse-transcription. Three µg of isolated total RNA were used for reverse transcription (RT), which was performed with Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, KRD, Prague, Czech Republic), oligo (dT)21 was used as the primer.

Real-time polymerase chain reaction. The primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA estimation have been published previously (22): the downstream primer was 5'-TCTGGAACTTCTGCTGTCTCAGTGG-3', the CEA upstream primer was 5'-TGTAGCTGTTGCAAATGCTTTTAAAGGAGAAGAC-3', the upstream primer 5'-CATCACCAGCAGTTTCC-3', and the product length was 380 bp. Primers for CEA mRNA estimation were those suggested by Gerhardt et al. (23), i.e. the CEA downstream primer was 5'-TTCCTCGTTCAGGCTCTCAGG-3', the CEA upstream primer was 5'-TGTAGCTGTTGCAAATGCTTTTAAAGGAGAAGAC-3', forming a PCR fragment of 160 bp. The CK20 primers were designed in our laboratory; the downstream primer was 5'-CTGATGCAGATTCGGAGTAACA-3', while the CK20 upstream primer was 5'-TCTCTCCTCCAGGTTGCTTAAC-3', giving an amplicon length of 162 bp. All the primers were designed to produce PCR products extended across at least one exon-exon boundary, so possible RNA contamination with genomic DNA would produce PCR products of considerably higher length than expected. All the quantitative estimations were done on a Rotor-Gene 2000 apparatus (Corbett Research, Sydney, Australia) in a total volume of 20 µl. In this volume, each sample contained 0.5 unit of Taq DNA-polymerase (Invitrogen, Life Technologies, KRD, Prague, Czech Republic), 1x manufacturer's buffer, 0.2 mM dNTP (each), 4.5 mM MgCl2, primers 0.6 µM each, and 0.5x Sybr-Green (Molecular Probes Inc., BioTech, Prague, Czech Republic). DNA standards for quantification were prepared by cloning individual PCR products into the pgEM-T vector (Promega Corporation, East Port, Prague, Czech Republic). All the cloned standards were verified by DNA sequencing using the ALFExpress sequencer (Pharmacia Biotech, Upsala, Sweden) and the Thermosequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Prague, Czech Republic). Plasmid DNA was linearized by a suitable restriction endonuclease, quantified spectrophotometrically and diluted to concentrations of 101-108 molecule copies per µl. The results are presented as absolute, i.e., the number of mRNA copies per 150 ng of total RNA, and relative, i.e., the number of copies of particular mRNA related to the number of copies of GAPDH mRNA in the same RNA preparation.

Serum tumour markers. Tumour markers in patients' serum samples were estimated in the Laboratory for Immunoanalysis, Faculty Hospital in Pilsen by routine techniques: CEA with the IRMA assay (DiaSorin, Sillwater, Minnesota, USA); carbohydrate antigen (TPS) with the IRMA assay (IDL Biotech AB, Bromma, Sweden); and tissue polypeptide antigen (TPA) with the IRMA assay (DiaSorin, Silswater, Minnesota, USA).

Statistical analysis. The SAS 8.02 software and STATISTICA 5.1 program were used. Standard statistical parameters such as mean, SE, range, median, interquartile range, minimum and maximum were calculated. For comparing different groups, non-parametrical tests (Kruskal-Wallis, Wilcoxon) were used because of the non-Gaussian distribution of the values. For correlations of different parameters within the group, the Spearman rank correlation coefficient was used.

Results

Non-cancerous control samples. The control group was comprised of 12 specimens of normal colon tissue (healthy margins), 14 specimens of benign lesions or colon inflammatory diseases, 11 specimens of benign liver lesions and 4 specimens of normal liver tissue. The obtained values from healthy tissues were compared statistically in all parameters with those of benign and inflammatory lesions,
and no significant differences between these 2 groups were found (p-values varied between 0.1572 and 0.9267). Therefore, both groups were used as a single control group in all statistical analyses. The combined control group represented 41 tissue samples from 29 patients with an age range of 18–78 years (mean 51.7).

**Differences in gene expression between tumours and controls.**

The main purpose of this study was to verify the usefulness of quantitative mRNA estimation of CEA and CK20 in tumour tissue. The obtained values are summarised in Table I. All the presented values show a significant difference between the tumour tissues and controls, as expected. Even if individual estimations gave good reproducibility in double or triple estimations (not shown), the overall distribution of the obtained values was extremely broad and did not show the Gaussian parameters. Therefore, the interquartile range and median, rather than the mean, characterized the particular group and the Wilcoxon and Kruskal-Wallis tests were used to compare individual groups. The CEA was expressed significantly in all CRC samples of the adenocarcinoma type, CK20 was, however, less specific and its expression was not detected in all specimens.

**Differences and correlations in gene expression between different tumours.**

Comparisons of gene expression levels between different types of tumours are summarised in Table II. A significant difference was found in absolute CEA expression between CRC and their liver metastases. Additional differences were found between CRC and other liver tumours, most of which were between liver metastases of CRC and other types of liver neoplasms.

Gene expressions in individual tissue samples exhibited a number of correlations, the best being between the relative values of CEA and CK20 in the group of other liver tumours, albeit a good correlation between those values was also found for the CRC group (Table III).

**Staging and clinical follow-up.** The TNM international classification was applied for the staging procedure. Among the CRC patients, 10 were evaluated as stage I, 31 as stage II, 24 as stage III, and 12 as stage IV. No significant differences were found in the CEA and CK20 expressions between the stage groups. On the other hand, the clinical 3-year follow-up after surgery revealed a very good correlation with the expectation based on staging. Briefly, the disease-free interval (DFI) showed a median value of over 3 years for stage I-III groups in the CRC group. In the liver metastases group, the median values in groups I-IV were >3, 2.26, 1.95 and 1.07 years, respectively. The overall survival in all cancers gave a median value above 3 years in stage I-III groups and 2.1 years in the stage IV group. The expression levels were also examined with respect to clinical development. All the cases were divided into 2 large subgroups for each expressed gene according to the mean value, i.e. one subgroup of those patients with expression levels above the average and the other subgroup with expression levels below the average. No significant relationship was found between the levels of CEA or CK20 expression and positive or negative clinical development in the 3-year period.

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### Table I. Absolute and relative values of specific mRNA copy number.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Colorectal cancer (n=85)</th>
<th>Liver metastases (n=75)</th>
<th>Other liver tumours (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR Median</td>
<td>IR Median</td>
<td>IR Median</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>GAPDH</td>
<td>4.2x10^4-6.3x10^5 1.4x10^5</td>
<td>5.5x10^4-4.9x10^5 1.1x10^5</td>
<td>7.6x10^4-5.6x10^5 0.0255</td>
</tr>
<tr>
<td>CEA</td>
<td>2.8x10^4-3.7x10^6 3.5x10^5</td>
<td>7.5x10^4-7.1x10^5 5.1x10^4</td>
<td>2.2x10^3-8.9x10^3 0.0012</td>
</tr>
<tr>
<td>CK20</td>
<td>0-7.8x10^5 4.5x10^5</td>
<td>1.3x10^5-3.6x10^4 1.9x10^4</td>
<td>6.1x10^2-2.9x10^3 0.0060</td>
</tr>
<tr>
<td>CEA/GAPDH</td>
<td>0.10-37.26 3.35</td>
<td>0.01-12.1 0.85</td>
<td>0-0.02 0.0184</td>
</tr>
<tr>
<td>CK20/GAPDH</td>
<td>0-6.42 0.01</td>
<td>0-3.87 0.17</td>
<td>0-0.01 0.0477</td>
</tr>
</tbody>
</table>

p-value refers to statistical significance (Wilcoxon test) of the difference between the given group and the control group (results not shown). IR, interquartile range; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; CEA, carcinoembryonic antigen; CK20, cytokeratin 20.

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### Table II. Significant differences in gene expression between individual tumours (Wilcoxon two-sample test).

<table>
<thead>
<tr>
<th>mRNA</th>
<th>CRC versus CRC liver metastases</th>
<th>CRC versus other liver tumours</th>
<th>CRC liver metastases versus other liver tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>0.0089</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CEA/GAPDH</td>
<td>0.0003</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>CK20</td>
<td>0.0380</td>
<td>0.0050</td>
<td></td>
</tr>
<tr>
<td>CK20/GAPDH</td>
<td>0.083</td>
<td>0.0083</td>
<td></td>
</tr>
</tbody>
</table>

CRC, colorectal cancer.
Correlations of gene expression and levels of tumour markers in serum. The results of standard tumour markers were available for some patients to the date of surgery and their serum levels were correlated with the mRNA expression in tissues. The available serum marker estimations were those of CEA, thymidine kinase (TK), tissue polypeptide antigen (TPA) and tissue-specific polypeptide antigen (TPS). The results are summarized in Table IV. No correlation between the CEA expression in cancer tissue and CEA levels in serum was found in the CRC group or even in the liver metastases group. In contrast, a good positive correlation between serum TPS and tissue CEA expression (absolute) in the CRC group and the liver metastases group was found. Other serum markers did not show any correlations with the measured gene expression.

It should also be stated that the pre-operative serum levels of some markers correlated with clinical development during the 3-year follow-up, namely CEA with DFI \( (p=0.0048) \) and overall survival \( (p=0.0005) \), CA19-9 with overall survival \( (p=0.0190) \) and TK with DFI \( (p=0.0082) \) and overall survival \( (p=0.0158) \), in the CRC group.

Histological types, grading and gene expression. There were 2 dominant histological types among the CRC and their liver metastases – adenocarcinoma and tubular adenocarcinoma. No significant differences in the expression of individual genes were found between these two types.

The examined samples were divided into groups according to the histological grading (G1-G4) and the distribution of gene expression parameters was evaluated using the Kruskal-Wallis test. The significant grading-dependent differences were found in CK20 expression \( (p=0.0443) \) for relative values in CRC, \( p=0.0095 \) for absolute values in the combined group and \( p=0.0275 \) for relative values in the combined group). The differences between any 2 grading groups were evaluated using the Wilcoxon two-sample test. Significant differences (Table V) were observed in CK20 expression, where the higher expression was typical for more differentiated tumours (lower grading forms), but even GAPDH was more expressed in the lower grade groups.

Discussion

RT-PCR remains the most sensitive method for the detection of specific mRNA and, in combination with fluorescence-based quantification, it became an important tool in the determination of mRNA levels in tissue. The external
standard system was used in each PCR run and the quality of the PCR product was verified by melting curve analysis. Each PCR reaction contained an equal amount of reverse transcription mixture representing 150 ng of the original total RNA. Therefore, our absolute values are related to the standard amount of total RNA. For normalisation, we chose the most popular house-keeping gene, GAPDH. During the analysis of our results, a significant difference in GAPDH expression was observed between tumours and control tissues (Table I). This is not unexpected since an increased glycolysis rate in tumours was observed eighty years ago by Otto H. Warburg (24) and, since then, many metabolic processes have been shown to be more intensive in tumours. The problem of normalisation was recently approached in more systematic studies, of which one group of authors recommended a panel of different standard genes (25), while the other authors (26) demonstrated the use of any standard gene for normalisation as meaningless and recommended the use of absolute values (i.e., copy number per Ìg of total RNA). Therefore, we have presented both forms of results.

The difference in gene expression between tumours and controls does not require comment. The differences between the different cancer groups could be a result of the characteristic behaviour of the individual cancer groups. Liver metastases of CRC differ in CEA and CK20 expression, despite their common origin. Liver metastases do not represent average CRC tissue, but rather clones of exceptional cells that succeeded in the metastastation process.

The most significant expression was that of CEA, which in CRC was present in practically all tissue samples. This is in contrast with the current belief that serum CEA is not elevated in all primary CRC or even in the metastases (Table IV). There was no correlation between CEA mRNA levels in tissues and those of the CEA marker in serum. Similar findings were described after CEA protein estimation in the cytosol of non-small cell lung cancer (14) and, more recently, in another lung cancer study where no correlation was found between the levels of serum CEA and CEA messengers in tissue (27).

Our results do not prove a significant relationship between cancer tissue CEA and CK20 expressions and staging, confirming results mostly reported for other tumours and estimated by other means (11, 12, 28). Our results are also in contrast with other publications (5, 6, 14) but, again, regarding different tumours and different methods. On the other hand, we were able to show the inverse dependence of CK20 (and CEA according to the Kruskal-Wallis test) expression on grading. It seems that CK20 (and CEA) is only expressed in tissues with a certain degree of differentiation. CK20 immunostaining is already used as a criterion in histological grading (29) in different tissues, but its detailed expression with respect to grading has not yet been studied.

The main conflict that remains is the absence of a correlation between the CEA gene expression and the CEA levels in serum. The details of CEA expression were studied earlier (3, 4, 30) in normal colon and colonic carcinoma and the existence of cell-bound CEA was demonstrated (4, 30), along with the free-circulating form. The two forms can even be distinguished with a specific monoclonal antibody (31). Circulating CEA is heavily glycated and its detection in serum might be dependent on the glycoprotein character of the particular determinants. Unfortunately, details regarding the antigenic determinants detected by the antibodies in commercial IRMA assay kits are not available (Immunotech Prague, private communication). In contrast, the CEA mRNA level in tissue is not connected with the glycation process and represents the instruction for both the cell-bound and circulating forms. It must be understood that the elevation of any serum marker, in principle, may indicate its increased synthesis and/or its increased "leakage" from the tissue into the extracellular space. It seems that the "leakage" is more connected with the tumour character and prognosis, since the prognostic relationship with CEA synthesis in tissue was not confirmed by the immunohistochemistry techniques (28). The mechanism responsible for the release of CEA into the extracellular space seems to be of importance and remains to be clarified.

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